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INVESTIGATION OF BIOFILMS IN COPPER TUBE CORROSION
AND THE SURVIVAL OF *LEGIONELLA PNEUMOPHILA* ON
ALTERNATIVE PLUMBING MATERIALS

AGSART
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A thesis submitted in partial fulfilment of the
requirements of the Open University
for the degree of Doctor of Philosophy

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Public Health Laboratory Service
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Porton Down, Salisbury in Collaboration
with Thames Water Utilities P.L.C.

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DEDICATION

To my mother and father and Gill

ABSTRACT

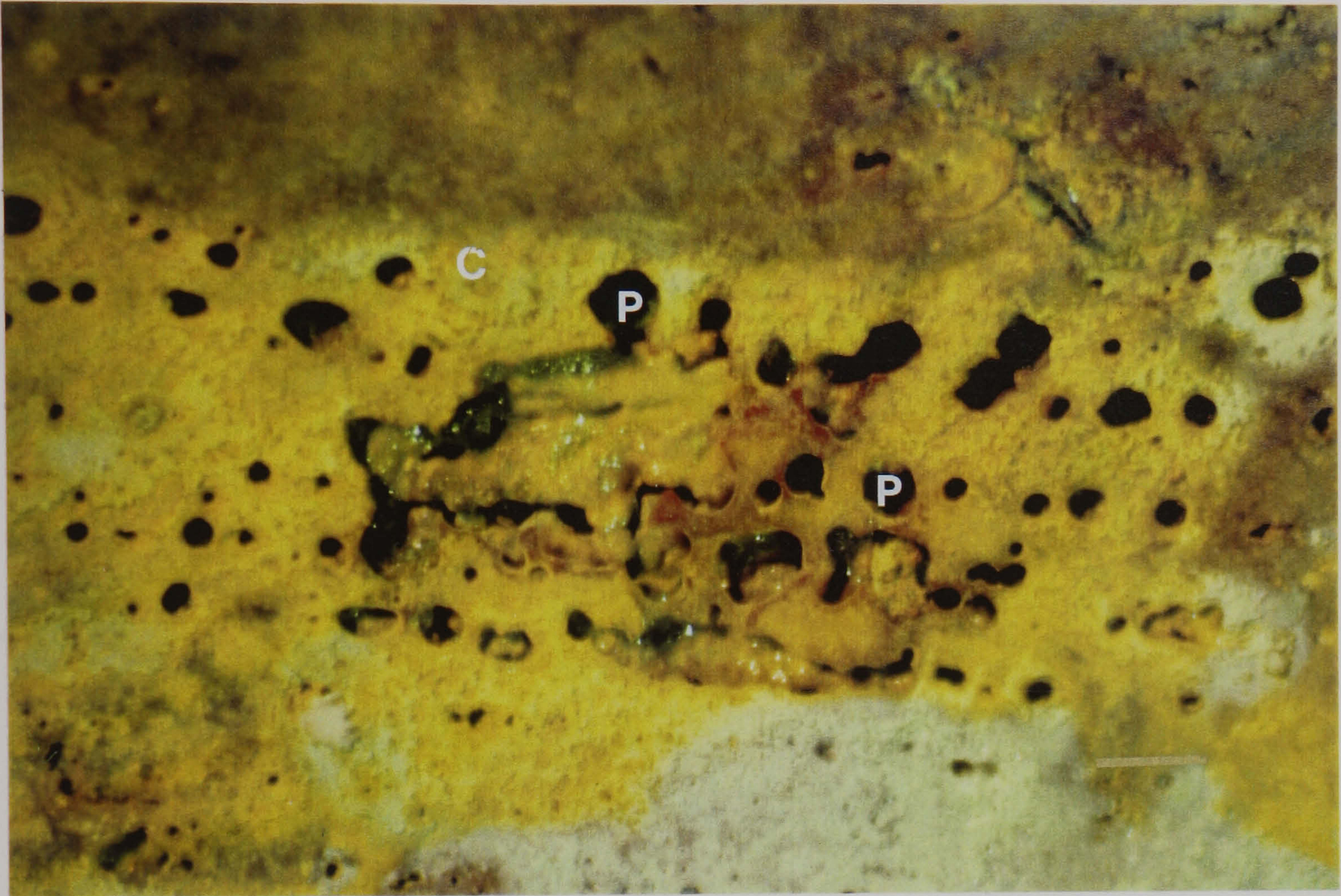
An unusual form of copper tube corrosion, occurring in two hospitals, was investigated during two site surveys and due to the presence of characteristic perforations became known as pepper-pot pitting corrosion. The corrosion was found to occur predominantly in soft water areas but mainly in hot water systems maintained below 50°C. When monitoring the hot water system at a particular site the water temperature was found to decrease overnight with a corresponding decrease in the dissolved oxygen concentration and assimilable organic carbon. Copious amounts of biofilm were recovered from the pipe surfaces thus it was hypothesised that metabolically active and respiring biofilm bacteria contributed to the creation of aggressive corrosive conditions at the copper tube surface. At control sites where this type of corrosion was not reported, the water temperature was found to be maintained above 50°C with reduced biofouling.

A laboratory model, using filter-sterilised potable water as the sole carbon source, was developed to investigate the conditions under which corrosion and biofouling was occurring. Biofilm development was demonstrated up to 55°C. At 60°C biofouling was very much reduced; however, a decrease in the number of bacteria recovered from the planktonic phase was only observed at 65°C. Planktonic bacteria were found to be dominated by pseudomonads while the biofilm was dominated by other Gram negative bacteria. Control measures that would slow down or prevent corrosion were studied. Pasteurisation (60°C) was found to prevent biofouling as well as controlling re-established biofilms but was less effective against consortia that had been previously exposed to this temperature. For the removal and control of biofilm, sulphamic acid was more effective than citric acid which allowed re-growth to occur within 14 days.

This unusual copper tube corrosion has resulted in increased use of alternative plumbing tube materials and therefore colonisation of copper and competitive plastic materials were investigated in the model system. Plastic materials were shown to encourage growth of *Legionella pneumophila* at 40°C whereas copper suppressed the growth of this water-borne pathogen. Results obtained in this investigation suggest that plastic plumbing systems pose a potential health risk by providing a means for transmission of pathogens such as *L. pneumophila*.

The association of biofilms with pepper pot pitting led to new ideas about mechanisms of microbially induced corrosion of copper tubing. A number of techniques including SEM, SCLM and light microscopy were used to demonstrate the heterogeneity and metabolic activity of biofilms produced in the laboratory model and on pipe surfaces. Mosaic microcolonies themselves are responsible for the initiation of differential concentration sites that are aggravated by exo-polysaccharides, metabolic activity and particulate matter in the aquatic environment. It is the localised distribution of initiated sites that could be responsible for the formation of multi-loci corrosion cells that are driven by an electrochemical potential forming the type of corrosion described as pepper-pot pitting.

Pepper Pot Pitting Corrosion on the Inner Surface of a Copper Pipe



Exposed view of etched copper surface illustrating characteristic pepper pot-pitting corrosion (Marker bar denotes 1 mm; C denotes the copper surface and P denotes the position of the pits).

Glossary of Terms

AOC	Assimilable organic carbon
AO	Acridine orange
CNS	Coagulase negative staphylococcus
cPVC	Chlorinated polyvinyl chloride
Cu	Copper
D	Dilution rate
DO	Dissolved oxygen
DVLO	Derjaquin, Verwey, Landau and Overbeek Theory
DIC	Differential interference contrast
EPS	Extracellular polysaccharide
ESEM	Environmental scanning electron microscope
ETS	Electron transport system
FITC	Fluorescein isothiocyanate
IFA	Immuno fluorescent assay
INT	Iodo nitro phenyl tetrazolium chloride
k_s	a constant, numerically equal to the substrate concentration
OGN	Other Gram negatives
Pe	Polyethylene
Pbu	Polybutylene
RITC	Rhodamine isothiocyanate
SCLM	Scanning confocal laser microscope
SEM	Scanning electron microscope
SPC	Standard plate count
SRB	Sulphate reducing bacteria
s	Limiting substrate
μ	Specific growth rate

V_A	Attractive forces
V_R	Repulsive forces
x	biomass

**"MICROBES CAN AND WILL DO ANYTHING;
MICROBES ARE SMARTER, WISER AND MORE ENERGETIC THAN
MICROBIOLOGISTS, CHEMISTS, ENGINEERS AND OTHERS"**

D. Perlmon. 1980. Dev. Indust. Microbiol. **31**.

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CHAPTER 1

INTRODUCTION

1.0 BACKGROUND

Since the late 1980's several large institutional buildings, such as hospitals, in very diverse areas (including Scotland, England, West Germany and Saudi Arabia) have experienced problems of copper tube failure. This has occurred in either the hot or cold water circuits, or in some cases both. After a series of site surveys in central Scotland to investigate the physico-chemical properties of sites experiencing copper tube corrosion it was tentatively proposed that bacteria could be involved in the corrosion process. Bacteria were discovered adhering to the inner surface of copper tubing in the hot water circuit where corrosion was occurring. Overnight, parameters such as temperature, assimilable organic carbon (AOC) and oxygen concentration fell markedly. These results suggested a hypothesis that, with a decrease in temperature, bacteria adhering to copper surfaces would become metabolically active, respire oxygen and consume AOC present in the water. Exo-polysaccharide produced by bacteria would chelate copper ions resulting in copper concentration cells. The heterogeneous mosaic structure of biofilm may serve to form local anodic and cathodic sites of corrosion as well as producing metabolic acids to aggravate the corrosion process. Hence biofilms have the potential to increase any physico-chemical corrosion which would have been taking place.

In order to investigate this hypothesis a laboratory model was established to simulate environmental parameters under which corrosion of copper tubing was occurring. Fouling of copper tubing, similar to that which had failed, was investigated within a continuous culture laboratory model where the media and sole carbon source was filtered sterilised water from the site of corrosion. The inoculum was composed of microorganisms scraped from the surface of a corroded copper tube. Environmental conditions in the laboratory model were then altered to investigate fouling of copper surfaces under parameters as close to those occurring in the environment. As this

system was simulating fouling within a hot water circuit of a large industrial building it was important to consider the survival of pathogenic microorganisms, in particular *Legionella pneumophila* in biofilms. *Legionellaceae* are responsible for causing legionellosis and water systems have been identified as an environmental reservoir for this bacterium (Stout *et al.* 1992). Colonisation of various typical plumbing materials e.g. copper, cPVC and polybutylene as found in water circuits were investigated to compare survival of these pathogens on different substrata.

Conditions and procedures for the growth of bacterial cultures in laboratories are many and varied. However, culture conditions provided in the laboratory do not necessarily reflect the variable environment of natural aquatic systems. The conditions to which bacteria are exposed depends very much on the particular environment, but generally will include fluctuations in light intensity, substrate concentration, temperature, oxygen concentration and water velocity (Stevenson, 1978). Nevertheless, bacteria are ubiquitous in the natural environment and are capable of adapting to changing parameters on a continual basis. Obviously where favourable conditions exist then growth will result, whereas if conditions are unfavourable then growth could be reduced or prevented. Continuous culture methodology has been extremely useful in the elucidation of substrate utilisation and competition between different species for one or more substrates in the dynamic conditions of the aquatic environment (Meyer *et al.* 1985).

Where growth conditions are favourable, but a substrate is not available, then bacterial growth will be limited. In aquatic environments the commonest type of bacteria are heterotrophs and reduced organic compounds may act as the limiting substrate for growth (Geesey, 1987; Geesey *et al.* 1987). Stevenson (1978) suggested that there are three major traits which determine growth in the environment. Primarily, metal ions, secondly substrate utilisation and finally attachment. For bacteria suspended in the aquatic phase Stevenson (1978) also considered dormancy to be an extremely

important physiological adaptation to ensure survival. Substrate availability for microbial growth in aquatic environments is generally very low, with between 1-15 mg l⁻¹ of carbon available (Roszack, 1987). The term oligotrophic has been used to describe bacteria that grow in a medium containing < 1 mg l⁻¹ of organic carbon supplied as a complex mixture of compounds, e.g. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* have been shown to grow in the presence of only 25 and 10 µg l⁻¹ (Roszack, 1987).

1.1.1 Bacterial Survival

So, what is the physiological state of bacteria in the environment? The rich complex media and specific conditions for isolation and growth of *Legionella* spp. in the laboratory are not present in the environment. Yet this pathogen is found surviving in a habitat very far from conditions that are supplied in a laboratory. It has been suggested that many environmental bacteria exist in a dormant form of which two phases are recognised to account for bacterial survival (Novitsky and Morita, 1976). Constitutive dormancy is the most widely recognised form where certain environmental triggers are associated with sporulation, such as the depleted concentration of available nutrients in triggering the formation of endospores in *Bacillus* spp. Secondly, there is exogenous dormancy, a condition in which development is delayed because of unfavourable chemical or physical conditions in the environment and is an innate property of cells under genetic regulation. The most obvious recognisable feature of this exogenous dormancy appears to be development of small cells, first reported by Novitsky and Morita (1976). These authors (Novitsky and Morita, 1977) also postulated the reductive strategy of survival i.e. an increase in cells numbers without an otherwise expected increase in cell biomass. Indeed there was an increase of up to 8-fold in cell numbers when a marine isolate was placed under starvation conditions. Such growth resulted in the production of a greater number of progeny, which were

smaller in size than the parent cells, with a corresponding increase in cell numbers enhancing the probability of survival. The ultrastructure of starved cells of *Arthrobacter* spp. was similar to non-starved parent cells; however, in starved marine vibrios a large periplasmic space was found to be present; the function of the periplasmic space is unknown. When introduced into fresh growth medium, the starved cells readily exhibited an increase in size and within 8 generations were indistinguishable from the parent cells. Laboratory cultures of bacteria exhibit standard growth phases of lag, log, stationary and death phases when inoculated into batches of suitable media (Veldkamp, 1970). However, observations by Novitsky and Morita (1976; 1977) indicated mechanisms by which the transition into death could be avoided by bacterial cells in natural environments so they could recover when favourable physical-chemical conditions were recurred.

1.1.2 Waterborne Microorganisms

Potable domestic water systems have been an important environment for study to scientists concerned with public health. From 1911-37, 16 out of 20 waterborne outbreaks in the United Kingdom resulted in enteric fever, with two being due to dysentery and gastro-enteritis. In 1937 a public enquiry implicated a water supply in a large outbreak of typhoid fever in Croydon, Surrey. It was this outbreak that led to routine chlorination at source of public water supplies. Since 1937 there have been 21 outbreaks associated with public water supplies, only one of which was identified as being due to paratyphoid (George *et al.* 1972). In 1944 Shannon and Wallace identified the importance of bacteria to water quality. Of 495 colonies on agar identified from the water phase, 448 were Gram negative bacteria such as coliforms, *Alcaligenes* spp. and *Pseudomonas* spp. Many microorganisms, such as *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Klebsiella*, *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Spirillum*, *Clostridium*, *Arthrobacter*, *Gallionella*

and *Leptothrix* spp. are present in potable drinking water (Geldreich *et al.* 1972). Generally, these bacteria are not considered to be pathogenic. However, *Flavobacterium* and *Pseudomonas* spp. have been known to act as opportunistic pathogens and *Pseudomonas aeruginosa* has been identified as a major causative agent of hospital acquired infections (Favero *et al.* 1971). In the United Kingdom a total of 15 outbreaks of disease were identified between 1977-86 (Galbraith *et al.* 1987) with *Campylobacter enteritis* and viral gastro-enteritis accounting for 66% of outbreaks. From 1987-90 there was 11 water-borne outbreaks with a shift from bacterial or viral enteritis to Cryptosporidiosis which accounted for 64 % of outbreaks (Stanwell-Smith, 1991).

Between 1971-78, 224 outbreaks of waterborne disease affecting 48,193 individuals were reported by 43 states in the United States of America (Craun, 1981). Reported outbreaks increased between 1971 and 1978 (as occurred in the UK between 1977 and 1986) which was attributed to more active surveillance; however, more outbreaks probably occur than are officially reported. In a report published in 1973, Craun and McCabe, estimated that only 50% of waterborne outbreaks in municipal water systems were reported. Indeed at least two epidemiologically linked cases of disease must be reported before an outbreak is declared and a common source noted and investigated. In only 45% of cases was the aetiological agent identified, predominantly *Salmonella* and *Shigella* as these bacteria were actively sought. The most commonly identified pathogen was *Giardia lamblia*, a flagellated protozoan responsible for giardiasis. Not all outbreaks were as a result of microbial contamination of the water supply. Chemical poisonings accounted for 10% of outbreaks, four of which involved acute copper poisoning involving concentrations of 4, 12.5, 38.5, and 80 mg l⁻¹ (Craun *et al.* 1976). The leaching of copper from water distribution systems was due to a number of factors such as a naturally aggressive water with a low pH, interruption of pH adjustment of a naturally aggressive water and a defective check valve on a drinks machine released CO₂ thus causing the water to become aggressive and leading to

dissolution of the copper. Acute gastro-enteritis due to chemical poisoning has also been reported in the UK. Metallic poisoning due to copper and zinc leachate has been reported (Anon, 1986). Phenol (Jarvis *et al.* 1985) and aluminium sulphate (Stanwell-Smith, 1991) have been implicated in separate outbreaks and were due to chemical spillage events.

During a comprehensive study of two water filtration plants, Payment *et al.* (1988) identified opportunistic pathogens such as *Aeromonas*, *Acinetobacter*, *Bacillus cereus*, *Flavobacterium*, *Moraxella* and *Pseudomonas spp.* (non-aeruginosa) in the untreated section of a water system. Primary pathogens identified included *Vibrio fulvis*. The treated water did not contain coliforms; however, *Aeromonas spp.* and *P. aeruginosa* were cultured from sampling sites in the distribution system downstream of the filtration plant. Such results typify the importance of regrowth in distribution systems where indicator organisms cannot initially be cultured. Actual numbers of indicator organisms may in fact be underestimated due to a variety of factors including excessive numbers of heterotrophic bacteria (e.g. with *Legionella*) and a process known as sublethal injury. This latter phenomenon was noted when coliform numbers from water containing chlorine were consistently higher by the multiple tube fermentation-most-probable-number (MPN) method than by a membrane filtration procedure. Bissonnette *et al.* (1975) suggested that more than 90% of indicator bacteria present in water systems may become injured in less than 1 week' exposure to chlorine. McFeters *et al.* (1982) suggested that injury sustained due to chlorine resulted in an inability to culture *Escherichia coli* from water samples and varying the diluent composition, temperature, time of exposure and growth media assisted in the recovery of injured bacteria. A major location of cell damage in water-injured coliforms is the cell envelope (Zaske *et al.* 1980) which may result in coliforms becoming sensitive to bacteriostatic or bactericidal compounds in the media. Sixteen different media normally used to culture bacteria from water suppressed growth with eleven that suppressed injured cells containing deoxycholate or bile salts (McFeters *et al.* 1982).

Where a problem of water contamination with coliforms or pathogens exists, the source has to be identified and dealt with such that no more cases occur. However dead end pipe sections and air chambers may be difficult to treat and as such would act as a foci of growth to reinoculate a water system if they were not found and remedied. Generally microorganisms in water systems can be controlled by approximately 0.3 mg l^{-1} residual chlorine, but even with remedial actions using 4.3 mg l^{-1} of free chlorine residual, coliforms may persist (LeChevallier *et al.* 1980). Ridgeway and Olson (1982) reported that free residual chlorine is an extremely potent bactericidal agent at concentrations of less than 0.1 mg l^{-1} but found that Gram-positive spore formers were able to survive 2 min exposure to 10 mg l^{-1} of free chlorine. Such resistance is an advantageous survival mechanism to particular bacteria.

Microorganisms are often associated with water/fluid systems. Bacteria can indirectly influence the consumption of potable water if their presence results in a deleterious effect upon materials used in the construction of water conduits resulting in odour or taste problems with water. Mains water has been documented to have discolouration and reduced flow through pipes due to microorganisms such as *Leptothrix* and *Gallionella* (Ridgeway *et al.* 1981). These chemolithotrophs use CO_2 in the oxidation of ferrous and manganese ions, resulting in precipitation of ferric salts around the stalked cells of *Gallionella*. Accumulation of ferric salts leads to discolouration and impairment of flow, and ultimately destruction of the support material infrastructure. Pipelines in a marine environment are normally made of mild steel and problems due to corrosion of those in use in the North Sea have been well documented (Hamilton, 1985; Edyvean, 1991). Costs of microbial induced corrosion to this particular industry have been very high.

1.1.3 Significance of pathogens in man made water systems

L. pneumophila sero group 1 Pontiac is the aetiological agent responsible for legionellosis. Legionellae have been recovered from many environmental samples including potting mix soil (Steele *et al.* 1990) and are thought to be ubiquitous in environmental water samples (Flierman *et al.* 1979 and 1981). Man made water systems such as potable water circuits, water circuit fixtures and fittings, storage tanks and cooling towers have all contained this family of bacteria (Dondero *et al.* 1980; Colbourne *et al.* 1984; Stout *et al.* 1985; Bhopal and Barr 1991). Legionellae are Gram-negative bacilli of approximately 0.3 - 0.5 μm by 2 - 3 μm . Amino acids in general play a major role in the growth metabolism of *L. pneumophila*, with no organic substrates except amino acids supporting growth (George *et al.* 1980).

Legionellosis is contracted through breathing in small water droplets. The majority of water droplets are large and will adhere to the nasal cavity lining but those which are 5 μm or less are able to enter lung alveoli (Baskerville *et al.* 1981). It is possible for aerosolised water droplets to contain bacteria and hence the aerosol will act as a means of transport for pathogenic bacteria such as legionellae. Once inside the lungs the bacteria are attacked by the primary host immune response involving macrophages. In situations where the host immune response is diminished, such as in herpes HHV6 infection there would be a suppression of lymphocytes which are the natural killer cells required in defence of a legionellae infection (Russler *et al.* 1991)

The disease, legionellosis, manifests as two forms. Firstly, there is pneumonia which is the more serious form and can be fatal, and occurs in persons who have an underlying predisposition e.g. the elderly, smokers, users of immuno-suppressive drugs or people with pulmonary conditions. With this form of Legionnaire's disease there is a 2 - 10

day incubation period and clinical features such as headaches, fever, cough, chest pain, diarrhoea and delirium and a mortality rate of 5-30%. Secondly, legionellosis also manifests as general flu symptoms known as Pontiac Fever. This is a self limiting, non-fatal influenza-like disease with a short incubation of approximately 3 days. Although, Pontiac Fever has a high attack rate the type of people affected have no underlying medical condition.

Many cases of legionellosis occur in hospitals, with hot water systems being implicated as the reservoir for *L. pneumophila* (Bartlett *et al.* 1983). Over the last decade a change in the maintenance of hospital hot water systems occurred which resulted in a reduction of the temperature from 60°C to between 40°C and 45°C. Although this change was initially introduced to conserve energy, it was later (in the USA) made a mandatory regulation such that, temperatures should not exceed 43°C to prevent scalding of patients in hospital wards (Joint Commission of Accreditation on Hospitals, 1981) - and a similar policy was followed in the United Kingdom. However, in 1983 the Joint Commission of Accreditation permitted each hospital to determine its own maximal hot water temperature.

Wadowsky (1982) grew *L. pneumophila* from waters between 25-37°C and found that at temperatures greater than 42°C the number of viable bacteria decreased dramatically. However, *L. pneumophila* has been recovered from hot water circuits in hospitals and hotels with temperatures greater than 42°C (Bartlett *et al.* 1983). Plouffe *et al.* (1983) recovered *L. pneumophila* sero group 1 from water storage tanks at 43°C to 45°C but not from systems between 58°C and 60°C. Also, in the last decade there have been a number of situations where the copper tubes in hot water circuits in hospitals have failed due to a previously unrecognised form of corrosion. In those hospitals, the hot water system was found to be maintained at less than 50°C and legionellae were recovered.

A number of factors within aquatic environments can influence *Legionellaceae* survival, such as cool spots in hot water circuits (Ezzedine *et al.* 1989), sediment in stagnant water e.g. the base of calorifiers where the temperature has decreased (Ciesielski *et al.* 1984), the presence of free-living amoebae which may protect the legionellae (Anand *et al.* 1983) and low chlorine concentrations in cold water systems (Kuchta *et al.* 1983).

Results from various investigators (Timbury *et al.* 1986; Colbourne and Dennis 1988) have established that *L. pneumophila* grows in biofilms on the surfaces of different materials in water systems. In order to eradicate this bacterium from water systems control mechanisms such as biocides have been utilised. Wright *et al.* (1991) demonstrated the efficacy of biocides to eliminate planktonic bacteria but more importantly that the biocides were unable to achieve a complete kill of attached bacteria. Thus resistance of attached bacteria to biocide treatment have suggested protective mechanisms. The presence of exopolysaccharides (Costerton, 1984) or slow growth rates (Brown, 1988) have been implicated as methods by which attached bacteria could maintain a resistance to antibacterial agents.

1.2 BIOFILM

Biofilm is a term used to define discrete aggregations of organisms, generally micro-organisms and their metabolic products at an air-liquid or liquid-solid interface (Ellwood *et al.* 1982). This usually involves bacterial contact with a solid surface within an aqueous phase resulting in microcolony formation (Marshall, 1976; Hamilton, 1983; 1985; Costerton *et al.* 1987). Aquatic environments can be thought of as a reservoir for bacteria which, given the opportunity, will form a biofilm. Bacteria have been found associated with surfaces immersed in both sea and fresh-water (Cooke, 1956; Wood, 1950), sand grains (Meadows, 1965 and 1971) soil

particles (Burns, 1980) and appear to be indigenous in all but the most nutrient-depleted environments (Costerton *et al.* 1987; Costerton and Geesey, 1979).

1.2.1 Mechanism of Attachment

Attachment of bacteria to surfaces is a very complex association involving many parameters including cell size, shape, physiological state, cell surface charge, conditioning pellicle of substratum, electrolyte concentration and flow of the aqueous solution. There are a number of stages involved in adhesion of micro-organisms to surfaces (Marshall, 1976): (i) Transportation of the bacteria to surfaces (ii) Deposition of organisms to surfaces, known as the reversible step which is essentially an instantaneous attraction (iii) Permanent attachment to surfaces is an irreversible sorption involving firm adhesion of bacteria with polymers acting as bridges between bacterium and solid substrata (iv) Colonisation of surfaces by growth of micro-organisms resulting in biofilm formation (Caldwell, 1983).

(i) Initial Attachment

When the attachment of a bacterium to a surface occurs then one has to consider that there will have been a preconditioning of that surface by organic and inorganic molecules in the aqueous environment. Therefore, a bacterium will probably not come into contact with a naked surface but the environmental conditioning layer or acquired pellicle (Fazio *et al.* 1982) derived from the aqueous phase.

The movement of a liquid is variable, depending on the distance from the substratum surface and may have an influential role in biofilm formation. When considering piped waters the velocity of a liquid in a tube will have a bearing on whether or not bacteria are able to come into contact with a surface, as the velocity differs according to the distance from the tube surface. Liquid moving near a surface moves slower than liquid

in the macro-environment due to drag caused by viscosity, resulting in a laminar layer. The laminar flow velocity at a surface was measured by determining the velocity of 0.2 μm latex spheres (Lawrence *et al.* 1987) and it was demonstrated that laminar flow velocity was more than two orders of magnitude less than the macro-environment. Terms used to describe the slow drag area include mass transfer resistance layer, hydrogen bonding layer and the viscous sublayer.

The relatively still or viscous sublayer next to a surface when an aqueous solution is under turbulent flow is thought to encompass a 40 μm zone (Characklis, 1981). It is in this viscous sublayer that inertial forces become relatively unimportant in comparison to viscous forces. As such, in order to become attached to a surface, bacteria must have the ability to penetrate this layer. Adsorption of soluble materials such as low molecular weight hydrophobic molecules (lipids and fatty acids) onto substrata may affect surface charge, surface free energy and adhesion as well as attachment of bacteria (Neihof and Loeb, 1974; Fletcher and Marshall, 1982). However, the presence of macromolecules in the aqueous phase may enhance, inhibit or have no effect on bacterial attachment (Meadows, 1971; Fletcher, 1976; van Loosdrecht *et al.* 1990). Fletcher and Loeb (1979) were able to demonstrate increased bacterial attachment to hydrophobic plastics with little or no surface charge e.g. polyethylene. Fewer bacteria attached to hydrophilic metals with a positive charge e.g. platinum and less to hydrophilic negatively charged surfaces e.g. glass. Considering that most bacteria appear to be polyanionic i.e. negatively-charged, then this latter negatively-charged, hydrophilic glass surface would have presented a most unfavourable substrata for them to attach to (Paul and Jeffrey, 1985).

In conjunction with the physical nature of the substratum, bacterial cell wall composition also has to be considered. Bacterial species can generally be divided into two main groups as to whether they stain Gram-positive or Gram-negative (Fig. 1.0). Both types of bacteria possess a cell wall and in Gram-positive cells this is relatively

thick (15-30 nm), rigid and composed predominantly of peptidoglycan. Covalently associated with peptidoglycan are other carbohydrate-type polymers, for example, teichoic acids and teichuronic acids (determined by growth in the presence or depletion of phosphate, respectively), polysaccharides and to a lesser degree, proteins. Protruding from the cell wall are amphiphiles, such as lipoteichoic acids which possess both hydrophobic and hydrophilic regions in their structure. Lipoteichoic acids have been shown to play a role in the adhesion of *Streptococcus pyogenes* to eukaryotic cells (Christensen *et al.* 1985). However, in Gram-negative cells the outer membrane is a complex multilayered structure of lipopolysaccharide proteins, phospholipids and lipoprotein. It is the lipoprotein which provides covalent linkage to the underlying peptidoglycan layer of the cell wall in Gram-negative bacteria. Gram-negative cells also possess polymeric amphiphiles, lipopolysaccharides, molecules which consist of three distinct regions covalently linked together: a hydrophobic lipid area (lipid A), a core polysaccharide and O-antigen-specific polysaccharide side chains. The lipopolysaccharide covers 30-40% of the outer cell membrane and is thought to be highly hydrophilic, thus rendering cells resistant to detergents and hydrophobic dyes. Bacterial cells like most eukaryotic cells are generally recognised as having a net negative charge (Richmond and Fischer, 1973; Rosenberg, 1981).

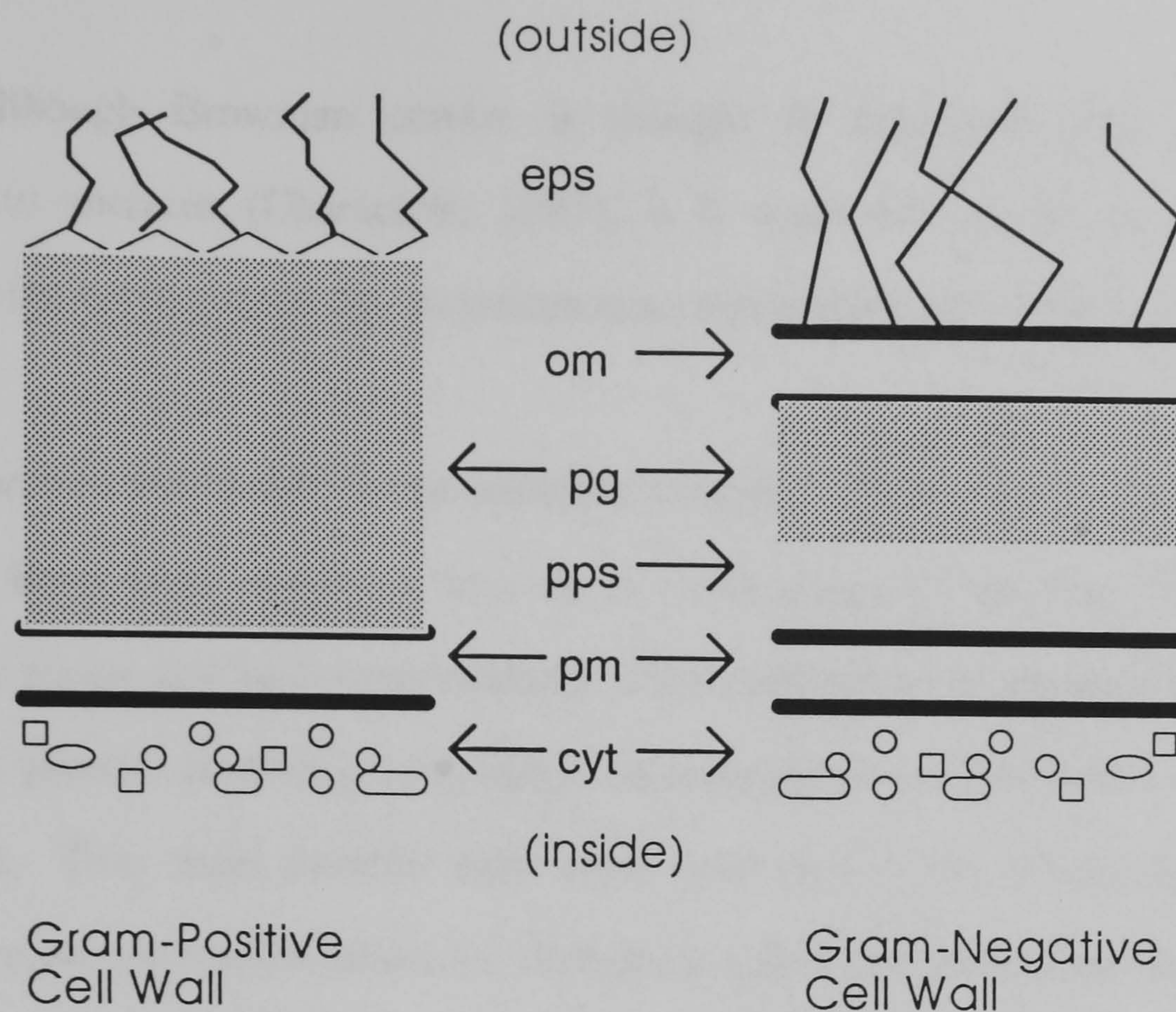


Figure. 1.0 Schematic diagram of Gram-positive and Gram-negative cell wall structures. eps denotes extracellular polysaccharides; om, outer membrane; pg, peptidoglycan; pps, periplasmic space; pm plasma membrane; cyt, cytoplasm.

A bacterium has to come into contact with a surface for attachment to occur, which implies some mechanism of transport. Under quiescent conditions transport of bacteria to surfaces is either by gravitational forces, Brownian motion or by motility, whereas if laminar flow is present then movement to a surface is by diffusion (Characklis, 1989). In a turbulent flow system, bacteria are primarily transported by fluid dynamic forces (Characklis, 1981) with gravitational effects being reduced as the density of a bacterial cell is only slightly greater than water (Marshall, 1985). Characklis (1981) suggested that sedimentation may not play a role in deposition of a single bacteria under the influence of turbulent flow. This was confirmed by Leech and Hefford (1980) who observed sedimentation of aggregates but not single cells of bacteria at the bottom of a capillary tube. Flagella provide bacteria with an active mechanism of transportation to a surface (Marshall *et al.* 1971) possibly displaying a positive chemotactic response to nutrient sources e.g. organic acids deposited as a pellicle on the surface (Fazio *et al.*

1982). Although Brownian motion is thought to contribute little to bacterial movement to surfaces (Characklis, 1981), it is considered to be of assistance in transport to the surface once the bacterium has entered the viscous sublayer.

Once a bacterium enters the viscous sublayer a number of basic forces and interactions occur and have been classified into three main groups: Van der Waals forces, electrostatic forces and hydrogen bonding. Initial adsorption of bacteria to surfaces is a reversible process involving long-range non-specific forces (Busscher and van der Mei, 1992). Two main theories have been used to describe interactions of small particles at close separation distances. Primarily, initial attachment has been described by a double layer phenomenon developed independently by Derjaquin and Landau (1941) and Verwey and Overbeek (1948) using lyophilic colloids to model living cells and known as the DVLO (Derjaquin, Landau, Verwey and Overbeek) theory. Basically all surfaces possess a charge and if the charges are similar then repulsion will occur. The DVLO theory states that the distance of separation between cell and surface in an electrolyte is the distance at which the repulsive (V_R) and attractive (V_A) forces are balanced (Fig. 1.1). An electrostatic double layer which forms around bacteria in an electrolyte consists of positive ions on the outside and negatively charged organic compounds on the inside i.e. extracellular polysaccharide. The difference in electrical potential across the electrostatic double layer is called the surface potential. Surface potential and electrostatic double layer thickness decreases with increasing electrolyte concentration. Therefore in high electrolyte concentrations the thickness of the electrolytic double layer and the surface potential are small, under which conditions, reversible bacterial adsorption readily occurs (Marshall *et al.* 1971). In the presence of sufficient electrostatic repulsion, a so called secondary interaction minimum occurs between approximately 10 and 20 nm. This theory considers long range forces such as London-Van der Waals attractive energies and electrical repulsion energies in the overlapping layers surrounding bacteria and substrata. London-Van der Waals attractive energies will tend to hold bacteria in the secondary minimum for a

short time. The mathematical expression of the DVLO theory of particle interaction includes the radius of particles. As the radius decreases the repulsive energy barrier decreases and attachment processes will be aided further by penetration of the primary minimum by cellular appendages. Examples of such appendages are fimbriae and lateral flagella that provide the intermediate structure between reversible and irreversible adhesion.

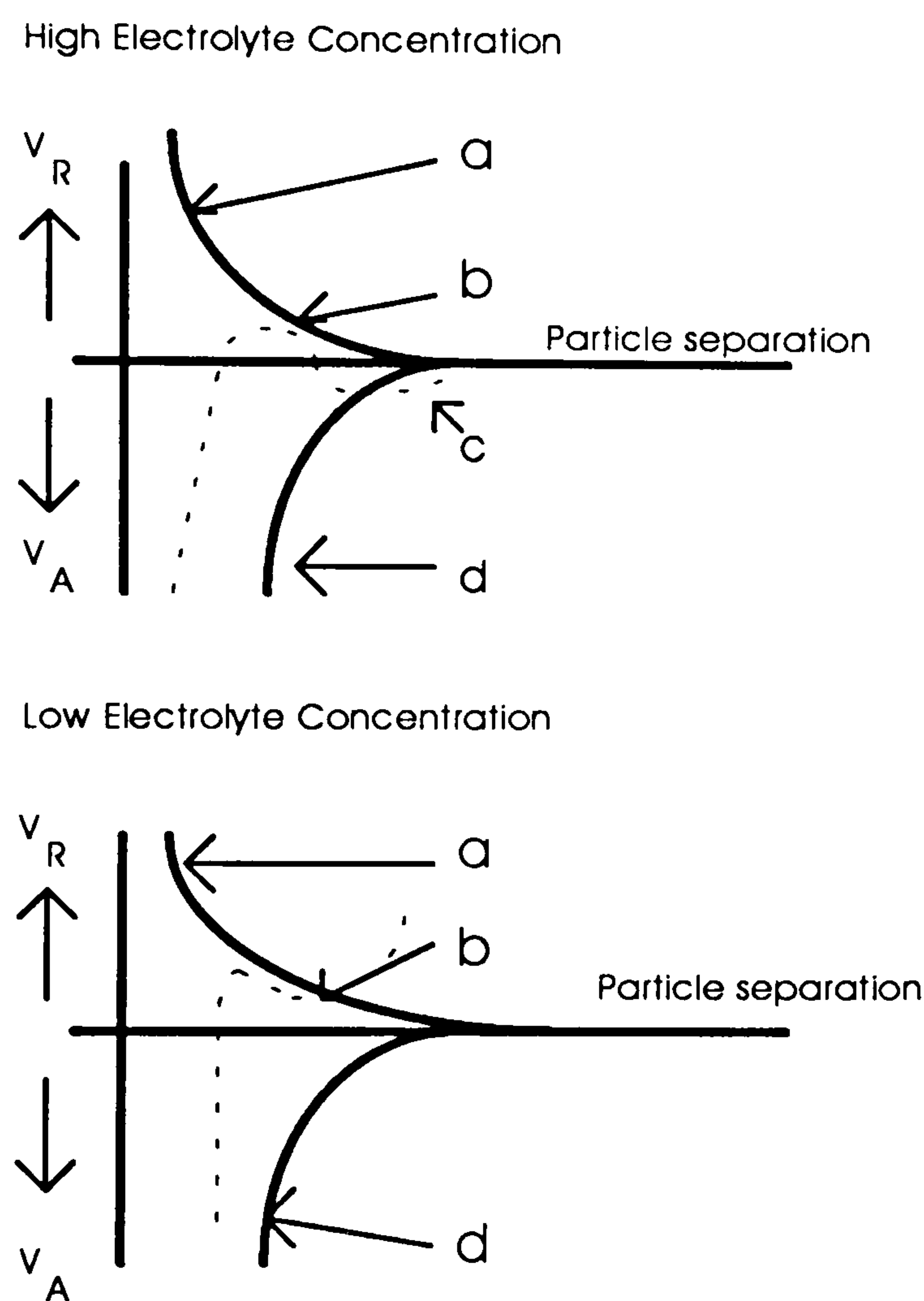


Figure 1.1 The interaction energy between a particle and substrate in a high and low electrolyte solution demonstrating the interactions at different separating distances. **a** denotes electrostatic interaction; **b**, secondary minimum; **c**, primary minimum; **d**, Van der Walls interactions ; V_R , repulsive forces; V_A , attractive forces (Dexter, 1975).

(ii) Irreversible Attachment

Irreversible attachment was found to be stimulated by extremely low concentrations of available carbon (Fig. 1.1). High concentrations inhibited this process which suggests

that low carbon concentrations, i.e. starvation, which produces cells of smaller radius, may in fact favour firm adhesion of micro-organisms to surfaces (Marshall *et al.* 1971). These specific short range bonds may occur over a relatively long time in respect to reversible bonding due to the necessary rearrangement of stereochemical and molecular groups by a cell in response to a surface. Ellwood *et al.* (1982) used Mitchell's chemiosmotic hypothesis of energy conservation (Jones, 1982) to suggest that microbial attachment could be energetically favourable to survival in low nutrient conditions. The translocation of protons out of the cell would generate a membrane potential (inside negative) which could be used to generate adenosine triphosphate (ATP), via an ATP'ase, as protons re-enter the cell. It is possible that contact at the surface enables cells to trap protons from an electrochemically active surface and/or surrounding cells, which it can use as an energy source hence stimulating growth and division. The former is a mechanism for enhancing a corrosion potential, while the latter is an example of inter-species hydrogen transfer. These are mechanisms by which a monolayer of dispersed cells have the potential to develop into a biofilm and may help to explain the ubiquitous nature of biofilms.

The presence of surfaces has been shown to result in a 25% increase in the growth rate of *Nitrobacter* cells attached to glass in comparison to those not attached to glass (Keen and Prosser, 1987). Increased growth was explained by the creation of a micro-environment low in nitrite (which is toxic at high concentrations) due to an extracellular slime layer formed by attached cells, rather than an accumulation of nutrients at surfaces. It is through the formation of polysaccharide adhesives that permanent attachment is mediated and has been described by Busscher and van der Mei (1992) as being a specific interaction. Christensen *et al.* (1985) identified two extracellular polysaccharides produced by a marine *Pseudomonas* spp. Busscher and van der Mei (1992) and Christensen *et al.* (1985) reported that polysaccharide B, only produced in the stationary phase of growth, was present as a capsule that was involved in initial adhesion by bacteria binding to surfaces (also indicated by Fletcher, 1988).

Polysaccharide A, in contrast was produced only by growing cells and may be produced after attachment irreversibly binds cells to the surface. Direct evidence for involvement of polymer bridging between bacteria and solid surfaces was obtained by sectioning at the adhesion surface (Fletcher and Floodgate, 1973). A number of studies of the effects that surfaces have had on bacterial activity are listed in Table 1.

Table. 1. Influence of solid surfaces on bacterial activity

Mechanism	Effect on attachment	References
Substrate uptake	stimulatory	Fletcher and (1979) Loeb
Number of cells attached	stimulatory	Hattori (1972/1981)
Change in cell size(starvation)	stimulatory	Kjellberg (1982)
Acid Production	variable	Ellwood (1982)
Respiration rate	variable	Bright (1983)
Heat production	diminished	Gordon (1983)

1.3 BIOMEDICAL ADHESION

1.3.1 Medical Implants

One of the areas where adhesion of bacteria can be a real problem is in polymeric materials used as medical prostheses for therapeutic purposes, such as catheters and implants (Jansen and Peters, 1991; Peters *et al.* 1982). Many materials used as medical implants are high molecular weight synthetic polymers such as polyethylene, polypropylene, polyurethane, polymethylmethacylate, polyesters, teflon or even silicone rubber. Foreign body infections involving strains of coagulase-negative

staphylococcus (CNS) have been identified as the main source of infections of catheters and prosthetic heart valves (Amoury *et al.* 1966). Other bacteria identified as major causative organisms of polymer associated infections are *Staphylococcus aureus*, *Enterobacteriaceae* and *Pseudomonas* spp.

Staphylococcus epidermidis is a ubiquitous commensal of human skin and mucous membranes, and although not normally causing disease in man it is an opportunistic pathogen. There are three main factors involved in determining whether it will cause disease i) strains which are encapsulated are more virulent ii) hosts who are immuno-compromised are more susceptible (as they are to other bacterial infections e.g. Legionellosis) iii) there is an increased incidence of infection in transiently implanted devices. Poisson *et al.* (1991) described microbial colonisation of endotracheal tubes in neonates who had been intubated at birth. A total of 14 tubes were examined and in half, the pharynx was found to have become colonised after three to four days of life and by a week in other cases. Significantly a slimy accretion designated to be of host origin predisposed to bacterial attachment of *S. epidermidis* and *Streptococcus agalactiae*. Although *Candida albicans* was isolated it was only found to colonise after 15 days.

S. epidermidis produces an extracellular slime layer of up to 120 μm thick on polyethylene catheters and also on an endocardial pacemaker (Peters and Pulverer, 1984). The slime layer, a glycoconjugate complex, was found to be loosely adherent to the organism and was easily removed when rinsed. This loosely adhered slime material interfered with the host immune response as the lymphoproliferative response of the human mononuclear cells (mostly T-lymphocytes) to polyclonal stimulators like streptococcal blastogen A was inhibited. Indeed, the slime layer was thought to present a mechanical barrier to antibiotic actions thus offering protection to encased bacteria, although Nichols *et al.* (1989) have shown that this is not always the case. It was suggested that slime layers may also prevent recovery of embedded bacteria during

culture thus resulting in false positives and hence lack of proper treatment for infected patients (Peters and Pulverer, 1984).

Despite host defence mechanisms and aggressive antibiotic therapy, bacterial biofilms on surfaces of plastic or metal prostheses are extremely persistent (Amoury *et al.* 1966). A number of strategies have been utilised to combat and or reduce bacterial infection of implanted polymers including the introduction of surface functional groups that exhibit anti-adhesive properties and the incorporation or coating of polymers by antimicrobial substances (antibiotics). The former method, involving modification of polymers with chemical radiation, was utilised to modify polyurethane (normally a hydrophobic surface) with the hydrophilic substance 2 hydrocymethylmethacrylate (Jansen *et al.* 1988). Thus, treated materials presented a hydrophilic surface to bacteria, resulting in reduced adhesion. In the absence of serum proteins, microbial adhesion is governed by hydrophobic forces and therefore less adhesion appears to result due to a weakening of hydrophobic interactions between the polymer and bacterium.

Infection of urinary catheters may result in growth of more than one species in the biofilm from the exterior towards the bladder (Marrie *et al.* 1983), while other intravenous or deepline feeding catheters (Hickman catheter) provide a conduit for growth of a single species biofilm of *S. epidermidis* from skin to the heart (Costerton *et al.* 1985). In many cases where aggressive antibiotic therapy has been utilised, bacteremia has been controlled but treatment has failed to kill the bacteria. Therefore, the bacteremia recurred when antibiotic treatment was discontinued (Marrie and Costerton, 1983; Peters and Pulverer, 1984). Due to such resistance, the only way to successfully treat biofouled prostheses is removal of the colonised device followed by parenteral administration of effective antibiotics.

The presence of biofilms does not necessarily result in a problem, as many intrauterine

contraceptive devices have been found to be coated with a thick multispecies biofilm yet patients from whom they were removed were not symptomatic (Costerton *et al.* 1985). However, in other instances where there are no clinical symptoms, biofilms are capable of profound effects such as decalcification of bone adjacent to colonised orthopaedic prostheses (Brause, 1989).

1.3.2 Dental Plaque

Another example of biofilms in the human body is that of dental plaque - the provoking agent of dental disease when the normal flora associated with health is perturbed. Plaque formation involves accumulation of bacteria in crevices between the tooth and gum (subgingival or supragingival plaque) and over the tooth enamel (coronal plaque). Formation of a biofilm results in i) the localisation of bacterial metabolites and toxic products resulting in dental demineralisation due to lowered pH (caries) and ii) toxic product formation causing mucosal inflammation (gingivitis and periodontitis). These diseases are among the most common of all infections in humans, with cost of treatment exceeding that of any other infectious disease (Gibbons and van Houte, 1992). Bacteria principally involved in causing disease due to dental plaque are the Gram-positive filamentous organisms streptococci (*Streptococcus mutans*), actinomycetes (*Actinomyces viscosus*) and Lactobacilli, Gram negative organisms such as *Bacteroides* species and *Hemophilus* species. As found with endotracheal tubes in neonates, a slime layer or accretion appeared to be a prerequisite for bacterial attachment (Poisson *et al.* 1991) in the oral cavity. An acquired pellicle of proteins adsorbs from saliva onto the tooth surface enamel, and ensures within seconds that the first contact between bacteria and tooth surface, over several minutes, is always via the pellicle (Belcourt, 1976). Although the initial adsorption layer (occurring within 10 s) was shown to be homogenous, by 2 h an uneven, knotted and heterogeneous structure characterised by small projections of organic material with a thickness of up to 600 nm was present (Busscher *et al.* 1989). It is the adsorbed pellicle components that

function as receptors for bacteria but bacteria also adhere to themselves creating a multiplicity of interactions. Many specific interactions of attachment have been catalogued in the formation of dental plaque including: lectins which are carbohydrate e.g. *S. mutans* glucosyltransferase; receptor proteins, fibrillae and salivary agglutinin (Christensen *et al.* 1985). Indeed formation of dental plaque provides the best example in human microbiology of bacteria employing intermediary bacteria to link themselves to tissue surfaces involving bridging ligands derived from the host (London *et al.* 1989).

Interestingly, it is evident that the degree to which a bacterium may attach to a surface influences the extent to which it may colonise. The mouth is only one of several environments in humans that are subjected to fluid flow and others include the intestinal canal, surface of the eyes and bladder. For a thriving bacterial community to survive in such situations bacteria must multiply at a rate exceeding the dilution of flowing secretions or alternatively bacteria must attach to survive. With swallowing of saliva occurring every few minutes, then attachment to the teeth enamel or epithelial cells must occur for bacterial growth to occur. Swallowing can be considered to have numerous functions, one of which is to wash bacteria from the surface of the teeth and so away from the mouth area. Turnover of epithelial cells has been found to be slower in germ-free rodents than in conventionally infected rodent mouths (Abrams *et al.* 1963). The highest turnover of cells in the mouth has been found to occur in the gingival epithelium which is closest to the bacterial mass or dental plaque, indicating that a plaque infested mouth encourages the turnover of epithelial cells (Gibbons and van Houte, 1992).

In summary, bacterial adhesion to surgical implants and prostheses are just a few of the areas where biofilms are a major complication in the treatment of chronic or critically ill patients. Catheter-related septicaemia represents the most frequent life threatening complication of vascular catheters (Raad and Bodey, 1992). Dental plaque formation

is also a detrimental process leading to the destruction of enamel and caries in periodontal disease resulting from a biofilm.

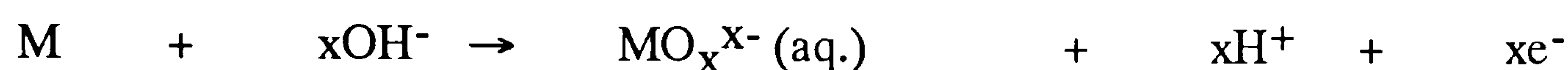
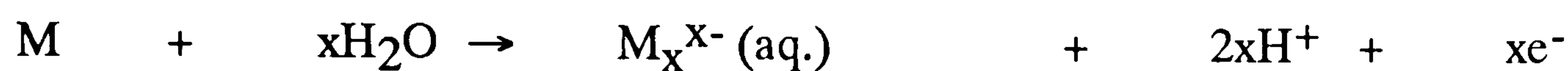
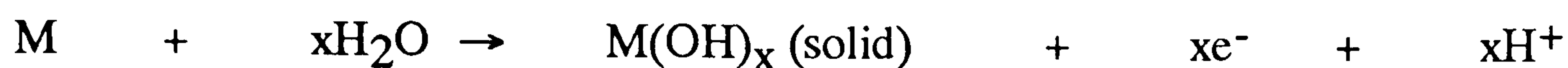
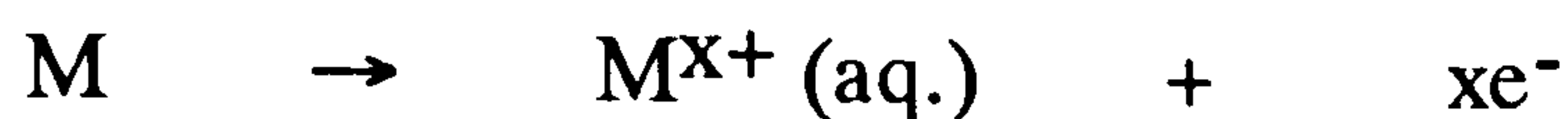
1.4 CORROSION

Biofilms can be beneficial or advantageous to man such as in the treatment of domestic and industrial effluent, biotechnological reactors (e.g. the use of immobilised cells for the production of microbial enzymes; Stanbury and Whittaker, 1984). However, the detrimental effect of biofilms can also be of industrial significance, including the loss of heat transfer and fluid flow, corrosion and general deterioration of equipment. Bacteria that have been mainly implicated in corrosion processes are the obligately anaerobic, sulphate-reducing bacteria (SRB). Problems in the offshore oil and gas industry due to these bacteria include (a) corrosion, either externally on pipe work and steel jackets or internally in water injection systems, (b) souring, of oil or gas due to production of hydrogen-sulphide, (c) plugging of reservoirs due to biofouling (Hamilton, 1983).

1.4.1 Chemical Corrosion

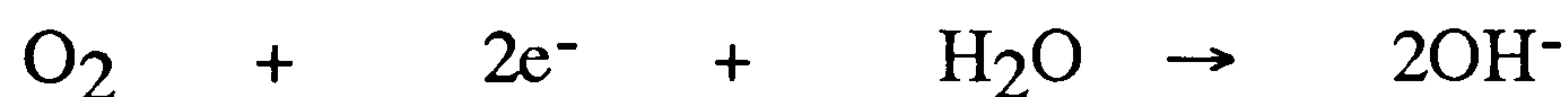
Metallic corrosion is an electrochemical process which is initiated by the presence of an impurity on surfaces such that a corrosion system is really a short-circuited electrochemical cell where pure and impure areas have come into contact with an electrolyte. Potential differences developing between pure and impure areas will cause an electric current resulting in corrosion. Sites at which oxidation of the metal atoms occurs is termed the anode. The cathode, where reduction takes place, is where the electrons are taken up. The tendency for a metal to corrode is determined by the electromotive force (emf) between the anodic and cathodic sites on the surface of the metal.

The following anodic reactions of metals (M) in the presence of aqueous electrolyte will occur in an environment free of biofilms with complexing or precipitating ions.



If corrosion product accumulates at the anode, the emf of the reaction decreases until corrosion ceases. This is known as anodic polarisation.

Where neutral aerobic electrolytes are present at the cathode (cathodic polarisation) the product is continuously removed from the cathode allowing the reaction to proceed.



In the above case, oxygen is the depolariser and this reaction will be the rate controlling step. Cathodic reduction of dissolved oxygen may result in an increase in pH of the solution at the metal surface. At the anodic site the metal will form cations which can be hydrolysed by water to form H^{+} ions.



Crevice corrosion, pitting, and erosion-corrosion are typical forms of localised

corrosion where the cathode/anode area, differential aeration and pH changes at anodic and cathodic sites are all important factors. For localised corrosion to occur there must be a continuous supply of electron acceptor species at the cathode such that the anodic reaction is not suppressed.

1.4.2 Microbial Corrosion

In general, a metal surface is able to corrode through having local anodic and cathodic sites. These can occur through heterogeneity in the metal surface; for example, if the surface is bathed in a solution which alters nutrient concentration (e.g. oxygen) from one area to another or the presence of films such as carbon. The area with the lowest oxygen concentration will release electrons, forming the anode, and the area of high oxygen concentration will consume electrons, becoming the cathode. This type of concentration cell is known as a differential aeration cell and can indirectly occur through the presence of a microbial microcolony on a surface.

Fouling is generally recognised as a layer of slime and deposits on a surface, as observed in cooling water storage tanks or barnacles and molluscs on piers stations or on the hulls of ships. Macro-deposition is unsightly and in the case of ships can create a drag effect resulting in increased fuel consumption to maintain the same speed (Copenhagen, 1966). Eukaryotes such as fungi have also been identified to be involved in corrosion of fuel tanks as they are capable of utilising diesel and jet aircraft fuel (Smith, 1991). In the 1960's, *Hormoconis resinae* was found to survive in fuel tanks of jet aircraft due to the presence of water as a product of condensation and growth resulting in structural damage (Miller, 1981). Fouling and damage was subsequently controlled by biocides and preventing the influx of water. Historic and artistic monuments in Spain have recently been monitored for attack and numerous fungi such as *C. cladosporoides* and *Penicillium chrysogenum* have been found to be

present (Flores *et al.* 1992)

Sulphur bacteria utilise sulphides and sulphur in the presence of oxygen to produce sulphuric acid. Although they have been a major problem in polluting surface waters in mining areas their ability to acidify toxic metals by dissolution of metal ores has been used to increase copper production from low grade ores. Such acid producing bacteria are especially active against carbonate rocks such as limestone and marble. Nitrifying bacteria are also able to attack carbonate rocks by the oxidation of ammonia to nitrites and the second stage nitrifactors to oxidise nitrous acid to nitric. (Petushkova and Lyalikova, 1992). Such bacteria are chemolithoautotrophs that obtain energy from oxidation of reduced sulphur and nitrogen compounds and use carbon dioxide as a single source of carbon.

(i) Sulphate-Reducing Bacteria (SRB)

Where acidic or anaerobic conditions prevail then protons take over the role of oxygen by accepting electrons resulting in the formation of first atomic then molecular hydrogen.



Under anaerobic conditions, bacteria such as *Desulfovibrio* spp. are able to utilise H_2 as an electron donor to reduce sulphate and achieve cathodic depolarisation by being directly involved in the corrosion process.

A classical mechanism of anaerobic corrosion was proposed by von Wolzogen Kuhr and van der Vlugt (1934). Cathodic depolarisation occurs through metabolic oxidation of hydrogen by anaerobic bacteria such as SRB. The mechanism of cathodic depolarisation is explained below in terms of chemical equations.

(1) Anodic reaction	$4\text{Fe} \rightarrow 4\text{Fe}^{2+} + 8\text{e}^-$
(2) Dissociation of water	$8\text{H}_2\text{O} \rightarrow 8\text{H}^+ + 8\text{OH}^-$
(3) Cathodic reaction	$8\text{e}^- + 8\text{H}^+ \rightarrow 8(\text{H})$
(4) Cathodic depolarisation by bacteria	$\text{SO}_4^{2-} + 8(\text{H})^+ \rightarrow \text{S}^{2-} + \text{H}_2\text{O}$
(5) Corrosion products	$\text{Fe}_2 + \text{S}^{2-} \rightarrow \text{FeS}$
(6) Corrosion products	$3\text{Fe}_2 + 6\text{OH}^- \rightarrow 3\text{Fe}(\text{OH})_2$

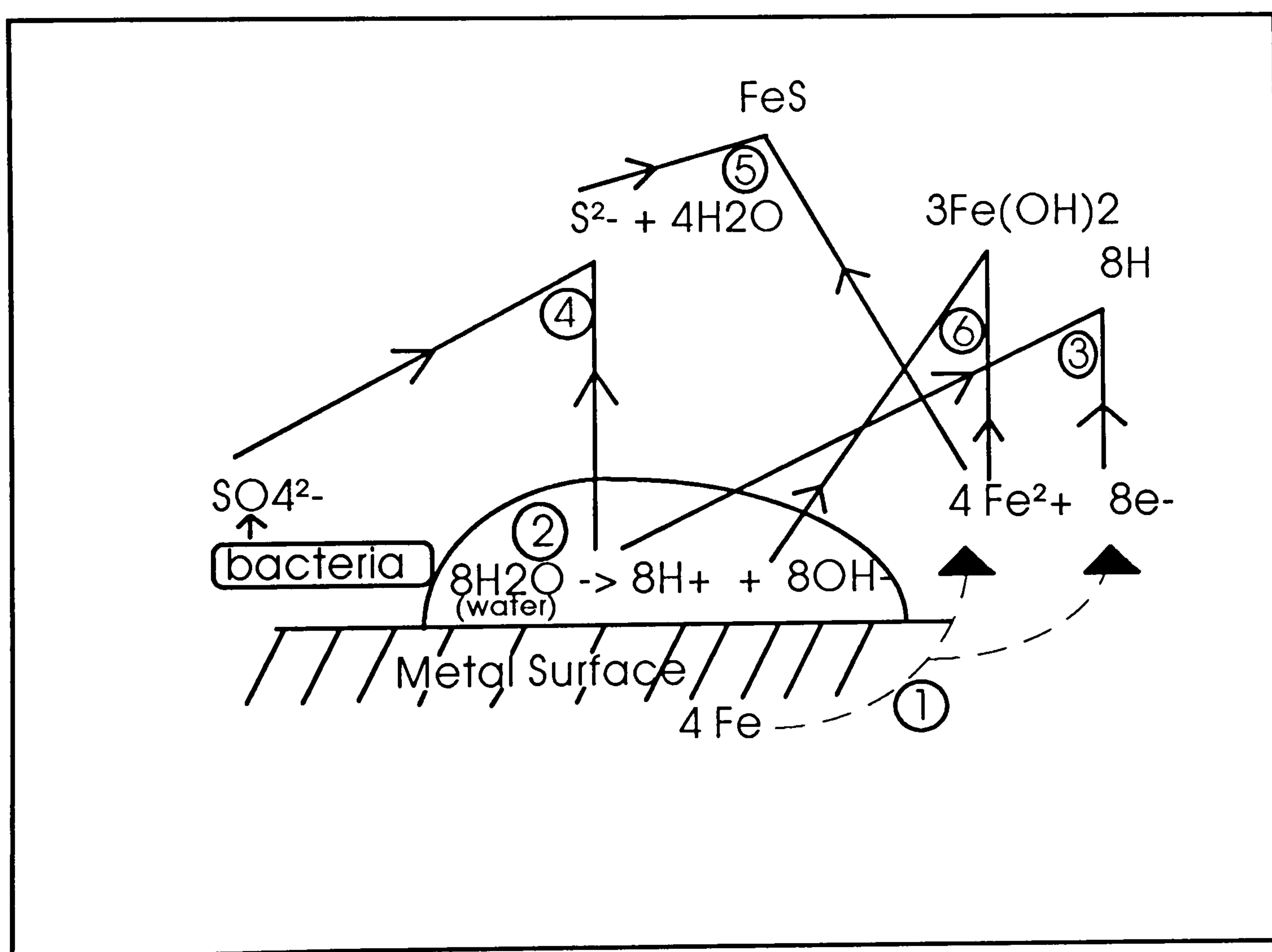
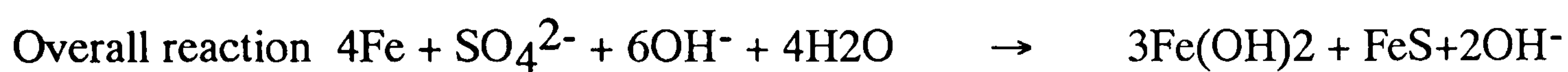


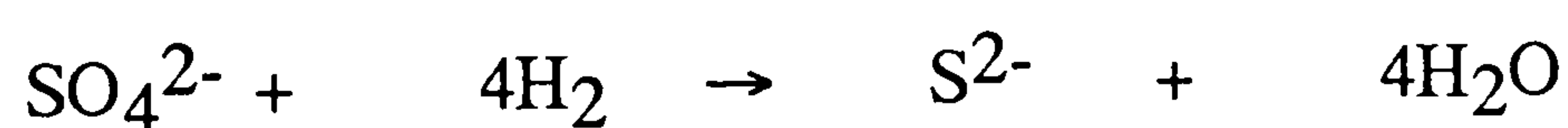
Figure 1.2 Schematic diagram of reactions (1) to (6) involved in the mechanism of cathodic depolarisation described above.

King and Miller (1971) later proposed that corrosion is under cathodic control with the critical electrochemical reaction being the adsorption of atomic hydrogen by ferrous sulphide corrosion products. Ferrous sulphide, not being a permanent cathode, is dependant upon the removal of hydrogen carried out by the action of bacterial hydrogenase located in the periplasmic space (Odom and Peck, 1984).

Other mechanisms may involve aerobic/anaerobic interfaces where sulphide could react with oxygen to produce highly corrosive elemental sulphur or polysulphides (Hardy and Bowen, 1984) - indeed these authors were able to produce a 90-fold increase in corrosion of mild steel when sparging an anaerobic culture of *Desulfovibrio* spp. with oxygen. Such varying conditions of aerobiosis would be more representative of conditions in environmental water systems where the concentration of oxygen at the aerobic/anaerobic interface is constantly or intermittently being altered.

SRB are also known to cause corrosion by production of highly corrosive and volatile phosphorous compounds believed to enhance dissolution of metal under anaerobic conditions (Iverson and Olson, 1983). Therefore, as well as the major corrosive product of FeS, SRB also produce highly corrosive iron phosphides (Fe₂P).

Therefore, SRB either play an important role in the overall corrosion processes by depolarisation of the cathode due to their ability to oxidise cathodic H₂ or by producing sulphide as an end product of their metabolism i.e.



It is the possession of hydrogenase enzymes that enable oxidation of cathodic hydrogen. Several other species belonging to the families *Bacillaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and *Rhizobacteriaceae* also possess hydrogenase enzymes and are able to oxidise the cathodic hydrogen whilst

hydrogenase-negative strains can not. Direct evidence for oxidation of cathodic hydrogen by *D. vulgaris* was provided by using a sensitive radio-respirometric assay (Hardy and Syrett, 1983) to measure production of ^{35}S -sulphide from ^{35}S -sulphate in the presence of an X-65 steel electrode. Growth on acetate and CO_2 as carbon sources only occurred if H_2 was provided as the sole terminal electron donor (Pankhania *et al.* 1986).

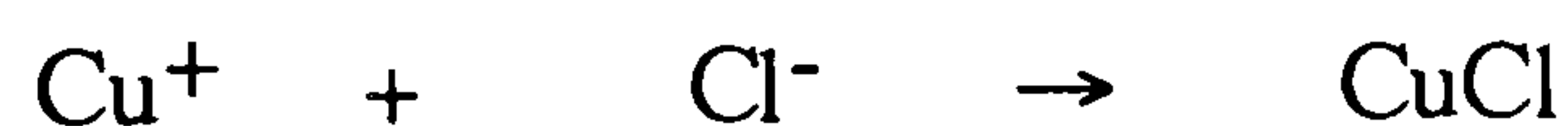
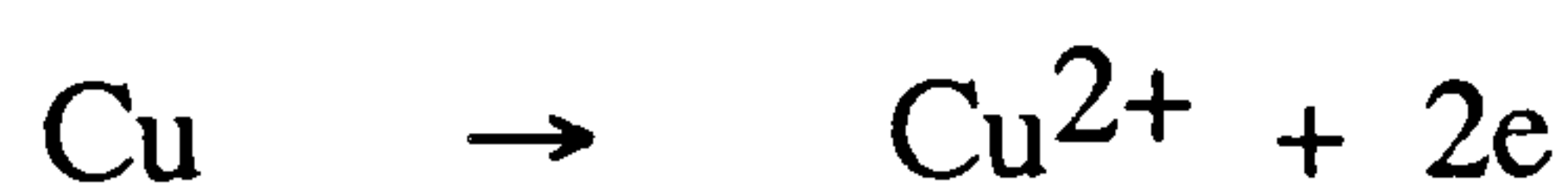
Many experimental studies using SRB in pure culture and in rich media do not necessarily represent *in situ* conditions. Indeed the dynamics of biofouling are such that one investigation of a coastal power plant demonstrated a total of 46 taxa from 6 phyla biofouling the surfaces (Edyvean and Videla, 1992).

Micro-organisms are known to cause corrosion by the following processes. (1) Absorption of oxygen and other nutrients by microbial growth adhering to damp or wet surfaces establishing concentration cells. (2) Degradation of lubricating oils that may act as a carbon source to support growth, causing a reduction of its protective effect against corrosion and wear (Smith, 1991). (3) Production of metabolic products (Prince and Morton, 1989): i) Sulphuric acid by *Thiobacillus*. ii) Carboxylic acids, which are corrosive to non-metals, but not known to be aggressive to copper. iii) Sulphide ions, produced by SRB. (4) In an anaerobic environment microorganisms such as SRB may remove hydrogen or form iron sulphides which further accelerate the cathodic process. (5) Production of extracellular polysaccharides.

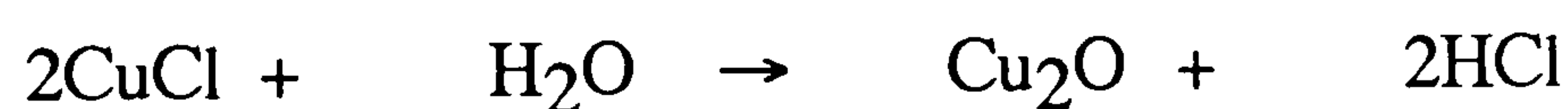
It is thought that all micro-organisms colonising a surface will produce extracellular polysaccharides (EPS) (Christensen *et al.* 1985) which collectively have been termed the glycocalyx (Costerton *et al.* 1985) largely composed of mannose, glucose and uronic acids joined by glycosidic linkages (Costerton and Geesey, 1979). The glycocalyx gel is able to immobilise water at the biofilm substratum interface and entrap metal species (e.g. copper, manganese, chromium and iron) and corrosion

products at surfaces. It has also been suggested to decrease diffusion rates towards and away from surfaces, as well as immobilise corrosion inhibitors and/or biocides (Hardy, 1989). However, pure metals themselves do in fact possess bactericidal properties which might reduce microbially induced corrosion (Bundy *et al.* 1980). Out of 16 pure metals, copper and cobalt were consistently observed to inhibit bacterial growth (Bundy *et al.* 1980).

In an aerated water supply containing chloride a clean copper surface will produce copper ions by anodic dissolution to combine with chloride to form cuprous chloride (Cornwall *et al.* 1973)



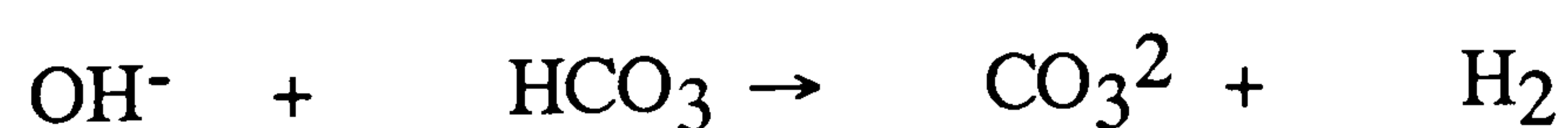
As cuprous chloride is unstable at near-neutral pH it hydrolyses to form cuprous oxide which precipitates at metal surfaces.



The cathodic reaction supporting the anodic dissolution process is that of oxygen reduction.



Removal of hydroxyl ions allows corrosion to occur which proceeds more readily in acid water supplies or in water containing bicarbonate ions.



This latter reaction results in the precipitation of a mixed calcium carbonate and basic copper carbonate scale. Precipitation of cuprous oxide at copper surfaces acts as an organic corrosion inhibitor having two main effects: Firstly there is formation of a protective layer that is protective at locally active anodic sites and therefore stifles attack, and secondly polarisation of the cathodic reaction. Corrosion of copper tubing has been classified into two types that are distinct in their characteristics.

Type 1 Corrosion (Campbell, 1950; Lucey, 1967), occurs in hard water on annealed or half hard tubes. Corrosion pits are hemispherical and chloride ions are to be found inside the pits with basic copper carbonates (malachite) as the corrosion product. This type of corrosion occurs only at temperatures less than 25°C.

Type 2 Corrosion (Mattson and Fredrikson, 1966) is recognised to occur in soft (pH less than 7.4, low bicarbonate to sulphate ratio), hot water (greater than 60°C) in hard-drawn copper tubes. Corrosion pits are deep and narrow with a copper sulphate (bronchantite) corrosion product.

Type 3 Corrosion (Shalaby *et al.* 1989) pitting is uncommon and occurs in pipes carrying cold water with a high pH, low hardness, low mineral and low organic content where the pits are characterised by small hemispherical pits under a common covering of basic copper sulphate.

Therefore corrosion Types 1 and 2 should not be confused, as they are distinct types of corrosion occurring in water of different chemistry and at different temperatures and none of the three types have been shown to be induced by microbial action.

Characteristically the type and rate of corrosion in hospitals in central Scotland could not be explained by what would be considered normal physico-chemical corrosion. Indeed water temperature (35-50°C), pH (7.5-9.0) and metallurgical condition of the

pipe indicated type 1 pitting, whereas water composition (soft water) and pit morphology (deep pits exhibiting pepper pot phenomenon) favoured type 2 pitting attack. A more detailed account of the corrosion found in the hospital water systems in central Scotland can be found in Chapter 3.0 (page 73).

1.5 CONTINUOUS CULTURE IN ECOLOGY

Bacteria present in the planktonic phase of a water system constitute a microbial ecosystem (microcosm) of which bacterial attachment to surfaces is a necessary extension. Ideally, a laboratory system should attempt to simulate as far as possible the conditions in the prevailing environment or part of the environment under consideration. However, in trying to accomplish this the natural microcosm will undoubtedly suffer as a result of being simulated in the laboratory. Whereas, microcosms allow a systematic examination of responses in natural communities to environmental manipulation, a model never actually aims to reproduce the entire system. A model seeks to examine properties of a part of the system, ignoring or holding all remaining factors constant.

In proposing to model environmental systems, the objective was to make the model as close to the natural ecosystem as is possible, such that the model is the system. To completely understand a particular ecosystem is probably less important than to discern rules of behaviour which only apply at a fundamental level to many different ecosystems. Two main views apply to the approach of studying systems. First of all there is the fundamentalist approach where each stage in the microcosm is studied, then pieced together and extrapolated to explain the whole system. Alternatively, an approach studying the whole system rather than the sum of the components could be termed the "holistic" approach. The reality, of course, is that both approaches are valid as at some point the holistic and the reductionist must interact to present a unified

explanation of the microcosm.

Aquatic systems actually have very few freely suspended individual bacteria. The microorganisms are usually associated with particulate matter, resulting in formation of bacterial rafts (Cohen, 1989). Most lakes behave like large fermenter systems where the annual cycling of thermal stratification is the only method by which mixing occurs (Wimpenny, 1982). It is at the interface of the warm less dense upper layers and the lower colder layer that activity of the bacterial population is greatest. Water temperature changes due to convective turnover as the seasons rotate, such that there is a shift in the stratification of water chemistry and bacterial population between the two layers.

In natural environments, many microorganisms are involved in cycling various elements. An understanding of individual components in the cycling process has been achieved by isolation of pure cultures and studying microorganisms under pure culture in the laboratory. Continuous culture has been used as it allows the study of microbial growth under conditions which can be similar to those in nature from where the inoculum was extracted.

Micro-organisms can be grown in open or closed culture conditions. Batch culture is an example of a closed loop system where growth continues until a limited amount of nutrient is used up and growth stops or adverse pH changes inhibit growth. This type of system is not generally representative of the environments found in nature except in the short term. Indeed, initially batch culture environments are substrate sufficient and have been successfully utilised to demonstrate growth in the presence of more than one substrate. This work has resulted in a detailed investigation of a number of cellular control mechanisms (Harder and Dijkhuizen, 1982). Where two carbon sources, particular if carbohydrate in nature, are present, bacteria will utilise substrates that will result in the greatest increase in growth rate while enzymes for other carbon source

will be repressed (catabolite repression). Sequential use of two carbon substrates and diauxic growth is the most pronounced response; however, both sequential use with no lag phase and simultaneous use have been observed using batch culture apparatus (Harder and Dijkhuizen, 1976).

Continuous culture systems involve the use of a culture vessel in which the culture is mixed and is continuously supplied with fresh nutrients at a constant volume. Spent culture and cells are continuously displaced with fresh medium such that growth can continue indefinitely. Such systems allow concentrations of one desired growth limiting substrate to be manipulated while all other factors remain constant. Other advantages of continuous culture systems are that they establish a reproducible steady state of growth, allow for the study of microbial responses to chemical and physical changes and contain a well analysed population of cells growing at submaximal growth rates typical of those which usually occur *in vivo* and *in vitro*. Other benefits are that nutrient concentrations can be as low as those found in natural environments. One caveat is that microorganisms rarely grow under true steady state conditions so such models need to be pulsed to mimic substrate fluctuations. A multi-stage system illustrating a two-stage continuous culture similar to that used in this study is shown in Fig. 1.3. An advantage of this type of system is that different conditions can prevail in different stages. In terms of this project, the laboratory model was operated to simulate an open continuous system such as that found in domestic plumbing. This type of vessel enabled coupons to be immersed into the culture such that attachment of bacteria to materials, as would happen on the inner walls of pipes in plumbing systems, could be monitored. Flow, as described by the Reynolds number (Characklis, 1981), within the vessel could also be manipulated to simulate the conditions within plumbing systems.

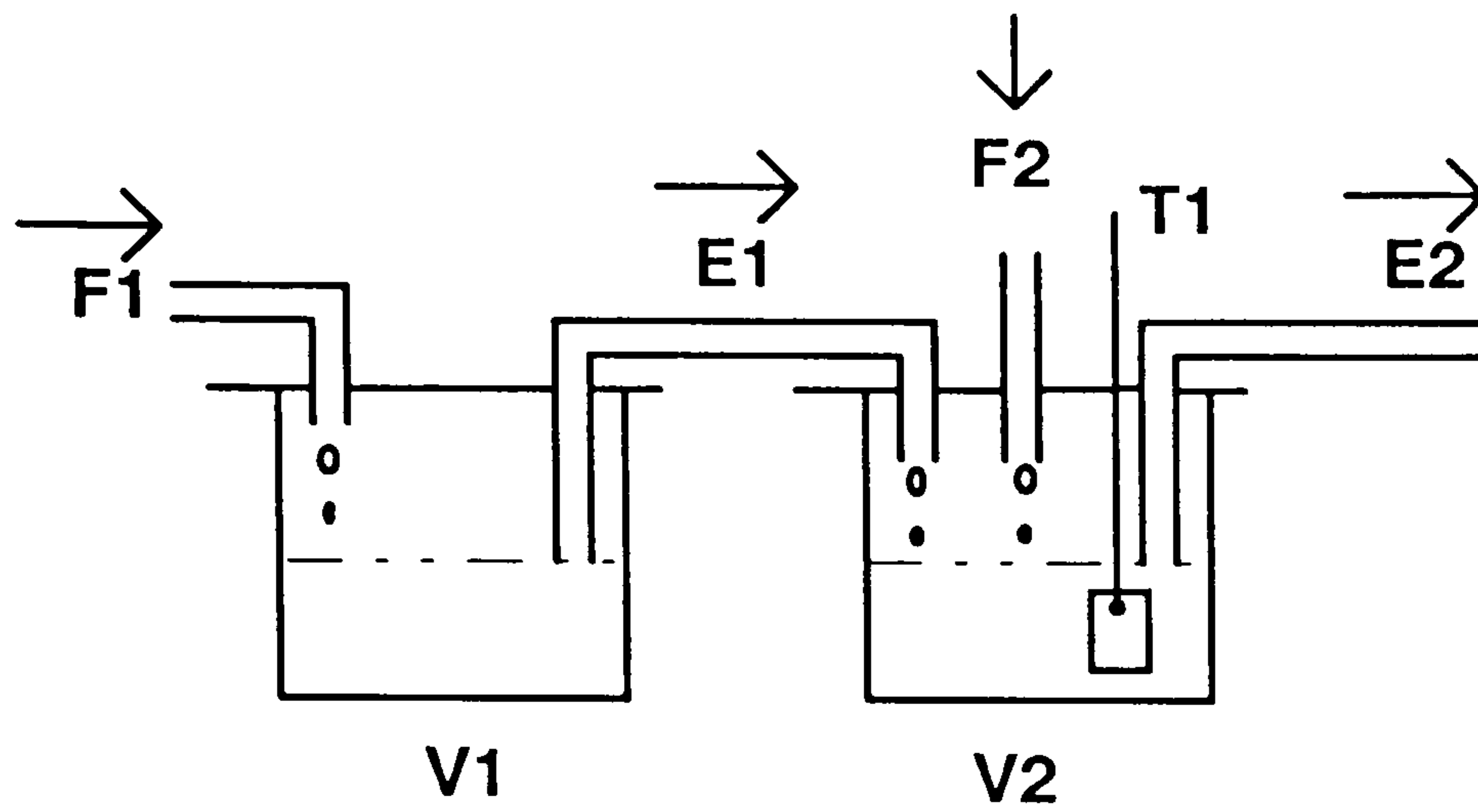


Figure 1.3 A multi-stage continuous culture laboratory model for biofilm formation.

V1, Vessel one; F1, Flow of sterile nutrients into vessel one; E1, Effluent of spent cells and media from vessel one which flows directly into vessel two V2 (F2); T1, Tile assembly on which to monitor biofilm formation; E2, Effluent from vessel two; F2, Flow of sterile nutrients into vessel V2.

In the chemostat, bacterial growth will be determined by substrate concentration, described by Monod as:

$$\mu = \mu_{\max} / (s/k_s + s)$$

Where μ is the specific growth rate per hour and s is the limiting substrate. This equation is based upon the assumption that the specific growth rate of a strain is related to its theoretical maximum (μ_{\max}) and is described by a Michaelis saturation equation involving substrate concentration; k_s is a constant, numerically equal to the substrate concentration at which

$$\mu = 1/2 \mu_{\max}.$$

In continuous flow, fresh medium is supplied continuously with the dilution rate D

(units of h^{-1}), being determined by flow rate divided by the culture volume. The combination of growth with dilution governs the change in the concentration of microorganisms in the vessel with time.

Change in biomass with time = growth - output

$$dx/dt = \mu x - Dx = (\mu - D)x$$

Bacterial numbers present in the culture will remain constant where $\mu = D$ i.e. $dx/dt = 0$ and a steady state exists. Where $\mu > D$ there will be a decrease in residual substrate concentration for the culture leading to a decrease in growth rate till $\mu = D$. Where $\mu < D$ an increase in substrate concentration will increase the growth rate resulting in a steady state culture unless D exceeds the critical dilution rate.

Although the term chemostat implies complete chemical control of the cellular environment, this is of course not possible. Difficulties of precision are compounded by inherent problems making it difficult to meet the assumptions used in deriving chemostat kinetics (Caldwell *et al.* 1992). Problems encountered include wall growth, back growth, plugging of tubing and also in obtaining complete mixing of the culture. Bryers (1984), used models to simulate biofilm formation which could also be modified to model mixed culture interactions, different suspended growth dependencies and various biofilm processes. He suggests that to neglect the presence of biofilms in reactor vessels can produce inaccurate estimates of kinetic/stoichiometric parameters and can lead to erroneous conclusions about the microbial culture under study, as part of the substrate would be used in biofilm growth. Model predictions suggest the metabolic activity of biofilms was greater than that of the freely suspended cells, indicating the importance of sessile bacteria in fermenters used to model suspended cultures. A number of different continuous culture chemostat systems have been devised, some of which are described below.

1.5.1 Laboratory model systems

A number of chemostat model systems have been devised to simulate environmental growth conditions of bacteria (Veldkamp, 1976). Single stage chemostats offer ecologically relevant information; however, they have the drawback that they are homogenous whilst most environments are actually heterogeneous. To simulate heterogeneous transient situations, models such as the multistage chemostat have been utilised which consist of a continuous flow culture system incorporating a number of linked vessels. Such systems have been used to model anaerobic ecosystems which are widespread e.g. aquatic sediments, waterlogged soils and sewage digesters. For example, anaerobic decomposition in a landfill includes anaerobic sulphate reduction, anaerobic nitrate reduction and methanogenesis, involving the sequential action of different bacterial types. Parkes and Senior (1988) therefore used a multi-stage chemostat to simulate an anoxic landfill dominated by methanogens with hydrogen as an important intermediate and acetogenic bacteria playing an important role in carbon mineralisation. Defined media were used. Heterogeneity within the model was increased by the addition of glass beads to increase surface area. However, in the progression towards simulation of environmental situations the increase in heterogeneity resulted in the system being less amenable to steady state kinetics. The system lends itself to modelling different environmental situations as required. The dilution rate of each vessel is dictated by the flow rate that will prevent washout or alternatively the volume of each vessel can be altered to control the dilution rate of individual vessels to accommodate for slow growing organisms.

To simulate temporal and spatial heterogeneity the bi-directionally linked continuous culture chemostat or the gradostat has been utilised (Wimpenny, 1988). The tubing-coupled gradostat consists of a series of five bidirectionally linked fermentation vessels with the culture transferred between neighbouring vessels in two directions at the same

time. Lovitt and Wimpenny (1981a and b) developed a diffusion-coupled gradostat in which vessels were linked by diffusion or mechanically assisted diffusion. Such a diffusion-coupled system has been used for the growth of *Methylophilus methyltrophus* with the highest cell yield occurring in the vessel nearest the methanol source. *Rhodopseudomonas marina* and *Rhodobacter capsulata* were also grown in varying concentrations of salinity. The diffusion-coupled system is an invaluable tool for the enrichment of different bacterial species where microbial consortia can interact even though some of the constituent species require mutually exclusive habitats.

Another system known as the compound bi-directional diffusion system has been developed by Lovitt (1981a). This is a three chambered system, each separated by a membrane to prevent physical interaction of bacteria but allows a diffusional interchange of substrates and metabolic products between the chambers. Herbert, (1992) utilised a 0.2 μm membrane with a reciprocating shaker to follow sequential flow of carbon through the microbial processes of a saccharolytic bacterium *Clostridium butyricum*, an SRB, *D. desulfuricans* and a purple sulphur bacterium, *Chromatium vinosum*. Glucose provided in the growth medium was metabolised by *C. butyricum* into lower fatty acids (primarily butyrate and acetate) and ethanol, resulting in a commensal relationship between this species and *D. desulfuricans* which utilised the primary fatty acids. The SRB was also able to enter into a mutual relationship with *C. vinosum* through cyclic reduction and oxidation of sulphate and sulphide (electron acceptor and donor respectively). Crump and Richardson (1985) using *E. coli* studied the growth of a sucrose nonfermenter and a strain that fermented glucose. Interestingly the two strains of *E. coli* were able to penetrate membranes with a pore size of greater than 0.1 μm within 24-48 h. Even with membranes with a pore-size of 0.1 μm the integrity of the membranes to bacterial cells was not maintained if the system was shaken continuously, perhaps illustrating the formation of ultramicrobacteria that penetrated the membrane or in severe cases, membrane rupture.

The above laboratory models have all been used to study bacterial growth in dynamic systems, and bacterial interactions in the planktonic phase, but they were not used to investigate bacterial attachment to surfaces. One of the main criteria that Caldwell *et al.* (1992) wished to satisfy was the definition of hydrodynamic conditions which require laminar flow velocity to be controlled. In order to do this he devised continuous flow slide culture cells which he considered gave greater precise growth conditions than chemostats. The flow cell consists of two cover slips over the top of two microscope slides resulting in a thin defined measured chamber through which the synthetic minimal media and cells flow. Subsequent review of that flow cell has suggested that due to its design that particular model may not actually provide controlled laminar flow velocity as previously suggested (Fowler, 1993). Automated computer microscopy analysis aided measurement of growth in continuous culture in terms of cell area. *P. fluorescens* has been shown to grow exponentially at a constant rate even within a single cell cycle while microbial growth during the formation of surface microcolonies was unbalanced. Colonisation manoeuvres associated with positioning, attachment and growth of this microorganism have also been observed (Lawrence and Caldwell, 1987).

Wimpenny (1982) was instrumental in developing another model for investigating bacteria in biofilms. In the constant depth fermenter, biofilm was developed within a recess of a predetermined depth with excess biofilm being mechanically swept away. Physically, the fermenter consists of (poly) tetrafluoroethylene PTFE with multiple recessed removable biofilm pans and a spring loaded PTFE scraper. Biofilm at a depth of 300 μm were generated using a dilute defined medium containing 50 mg l⁻¹ of carbon. This particular model has been criticised for a number of reasons. The culture consisted of a pure culture of bacteria, a constant 300 μm deep biofilm and a physical scraper impacting the biofilm have resulted in a system that is rarely found in the natural environment, perhaps resulting in an artefactual biofilm. However, the development of micro-electrodes in this work has been of immense value in the

understanding of parameter profiles within different layers of the biofilm (Wimpenny, 1988). For example, the use of oxygen electrodes with tips less than 10 μm thick showed how only the top 100 μm remains viable.

To study the influence of growth rate on the susceptibility of bacteria to antimicrobial agents, Gilbert *et al.* (1990) developed synchronous cultures of *Escherichia coli*. This was carried out by passing a pure culture of the bacterium through a filter, reversing the filter and collecting baby or daughter cells, as media was pumped through the filter to produce the synchronous culture. The effect of antibiotics on the mono-layer or biofilm-associated bacteria was then determined at different growth rates. Used as a method for separating and controlling growth rate, this model has demonstrated that the resistance of biofilm-associated cells relates to their slow growth rate rather than to any innate properties of the biofilm. However, further studies may have to be carried out to ascertain whether cells that have been impacted onto a filter i.e. biofilm-associated cells, behave and grow the same as bacteria which have formed a biofilm due to their own activities. Other investigators have described the growth of biofilms, in medical implants and laboratory models using clinically isolated bacteria, as a patchy layer of bacteria on the surface with flocculation's or fronds projecting from the surface (Marrie *et al.* 1982; Brooks and Keevil, 1993). If this is the case, then the biofilm-associated bacteria, impacted onto the filter as a mono-layer, may not represent what is happening in the environment, however, in controlling growth rate as a factor in antibiotic control of these biofilms then it does appear to be very efficient. As in all laboratory models the investigator has to be very careful in extrapolating results to a real environmental situation.

The systems discussed above are only a selection of the models and simulators which have been developed to suit the needs of the various investigators in their specific wish to investigate a particular environment. Each of the systems are defined by their operators to answer particular questions about the physiology of growth in the

planktonic phase, attachment or even interaction within formed biofilms.

When presented with the task of finding a system to model a water system the one selected was that used previously by Keevil *et al.* (1987). Although that particular model was used to simulate dental plaque it was modified from a single stage homogenous chemostat to represent a flowing system as found in domestic water systems.

CHAPTER 2

MATERIALS AND METHODS

2.0 IDENTIFICATION OF BACTERIA

Identification of the aquatic, non-fastidious bacteria was performed using colony morphology, pigmentation, Gram staining (Bacteria were heat fixed, stained with methyl violet for 1 minute, rinsed in sterile distilled water, stained with iodine for 1 minute and decolourised with acetone. The bacteria were then counter stained for 30 s with carbolfuscin before being dried and viewed using a light microscope), oxidase and catalase tests (Bergeys, 19). Further identification was also carried out on strains using API 20E, API 20NE identification kits and the Vitek identification system in conjunction with the API database of biochemical determinants (API Biomerieux, Basingstoke).

2.1 IDENTIFICATION OF *LEGIONELLA PNEUMOPHILA*

Colonies of suspected *Legionella* spp., characterised by a morphology of spherical colonies with a dark grey, almost blue with a glassy appearance, were subcultured onto BCYE and non-cysteine media for primary identification of *Legionella* spp. and incubated at 30°C overnight. Those cultures which grew on BCYE (and had a characteristic smell) but not on non-cysteine media were classified as *Legionella* spp. and bacterial suspensions were prepared in sterile distilled water (1 ml). 10 µl of the bacterial suspension was placed onto the wells of a multispot microscope slide (Flow Laboratories, UK). The slide was then dried at 37°C before being fixed in acetone. Each well was then coated with 10 µl of polyclonal antibody 1-14 (Thames Water, Plc.) and the well incubated in a moist atmosphere for 1 hour at 37°C. Following three washes in phosphate buffered saline (PBS) and drying at 37°C, each well was coated with 10 µl of anti-rabbit IgG FITC conjugate, and incubated for 30 minutes at 37°C.

The microscope slides were then washed three times in PBS and dried at 37°C before viewing with an immunofluorescent microscope (Nikon, UK Ltd). For further subtyping of the *Legionella* spp. colonies, subsequent wells were coated with type-specific monoclonal antibodies followed by anti-mouse IgG FITC.

2.2 MICROBIOLOGICAL ANALYSIS OF SITE SURVEY SAMPLES

Swabs of various taps, cold water holding tank and calorifier outlets were suspended in 10 ml sterile distilled water. All samples were serially diluted 10-fold in sterile distilled water with standard plate counts carried out in duplicate using a 0.1 ml inoculum. Total aerobic bacterial numbers were enumerated on minimal R2A (Reasoner and Geldreich 1985, Table 2, page 46) and BCYE (Pasculle *et al.* 1980; Table 4, page 48), whilst legionellae were also recovered on BCYE (Pasculle *et al.* 1980; Table 4, page 48) and GVPC (Wadowsky and Yee, 1981; Dennis *et al.* 1984a, Table 5, page 49). Sulphate-reducing bacteria were recovered on Modified Barr's medium (Pankhurst; 1971, Table 3, page 47) (pipe samples only). All agar plates were incubated at 30°C for 7 days with bacterial counts reported as colony forming units (cfu) ml⁻¹ (± 100 cfu ml⁻¹). *Legionella* spp. were initially detected on BCYE and typed using immunofluorescent labelling (Section 2.1). Bacterial identification was established by colonial morphology, Gram staining, oxidase reaction, catalase reaction and API identification kits (2.1, page 44). Sections of copper tubing were cut from the tube samples and prepared for SEM analysis (section 2.12.2, page 67). Glutinous slime from the surface of the copper tubes was aseptically scraped onto a glass slide for Gram staining.

2.3 CULTURE MEDIA

Minimal R2A medium agar (Reasoner and Geldreich, 1985; Table 2) was used to isolate microorganisms aerobically by incubating at 30°C for 7 days. Modified Barr's medium agar (Pankhurst 1971, Table 3), was selected for the isolation of sulphate-reducing bacteria (SRB), which were incubated anaerobically at 30°C and 40°C for 7 days. BCYE agar (Pasculle *et al.* 1980; Table 4) and GVPC agar (Wadowsky and Yee, 1981; Dennis *et al.* 1984a; Table 5) were used for the detection of legionellae and incubated aerobically at 35°C for 7 days. When the vessel temperature was at 60°C, 0.1 ml aliquots were plated on to modified Ramaley and Hixson (1970) medium agar (Table 6) and incubated aerobically for 7 days at 60°C for the recovery of thermophillic bacterial species. Methylobacterium medium was used for the recovery of C1 utilising methylotrophs (Table 7).

Table 2. Reasoner's Media

Ingredient	R2A g l ⁻¹	R3A g l ⁻¹
Yeast extract (Oxoid)	0.5	1.0
Difco proteose peptone 3	0.5	1.0
Casamino acids	0.5	1.0
Glucose	0.5	1.0
Soluble starch	0.51	1.0
Sodium pyruvate	0.50.3	1.0
K ₂ HPO		0.5
MgSO ₄ .7H ₂ O	0.5	0.6
Agar (Oxoid)	15.0	15.0

pH 7.7 and sterilise 121°C for 15 min.

Table 3: Modified Barr's Medium

Ingredient	g l ⁻¹	ml ⁻¹
K ₂ HPO ₄ anhydrous	0.5	
NH ₄ Cl	1.0	
CaSO ₄ .2H ₂ O	1.0	
MgSO ₄ .7H ₂ O	2.0	
70% Sodium lactate solution	5.0	
Ascorbic acid*	1.0	
Thioglycolic acid Na salt	1.0	
Oxoid yeast extract	1.0	
Tap water		1000.0
Oxoid purified agar	15.0	
Resazurin (1mg ml ⁻¹)*	1.0	
Na ₂ SO ₄	1.0	

* denotes modifications to the standard Barr's medium. Cool to 50°C, add 50 ml l⁻¹, 1% ferrous ammonium sulphate solution, pH 7.7 and sterilise 121°C for 15 min.

Table 4: Buffered charcoal yeast extract medium (BCYE)

Ingredients	g l ⁻¹ distilled water
Yeast extract (Oxoid)	10.0
Agar (Oxoid no. 3)	12.0
Activated charcoal (Norit SG)	1.5
α -ketoglutarate, monopotassium	1.0
ACES buffer (Sigma)	10.0
Potassium hydroxide (BDH)	2.8
L-cysteine	0.4
Ferric pyrophosphate	0.25

pH 6.9 and sterilise 121°C for 15 min.

Table 4.1: Non-Cysteine Medium (NCM)

Ingredients	g l ⁻¹ distilled water
Yeast extract (Oxoid)	10.0
Agar (Oxoid no. 3)	12.0
Activated charcoal (Norit SG)	1.5
α -ketoglutarate, monopotassium	1.0
ACES buffer (Sigma)	10.0
Potassium hydroxide (BDH)	2.8
Ferric pyrophosphate	0.25

pH 6.9 and sterilise 121°C for 15 min.

Table 5: GVPC (BCYE: minus KOH, plus the addition of the following)

Ingredient	g l ⁻¹	mg l ⁻¹
Glycine	3.0	
Vancomycin		5.0
Polymixin		11.0
Cycloheximide		80.0

pH 6.9 and sterilise 121°C for 15 min.

Table 6: Modified Ramaley and Hixson Medium (Thermus Media)

Solution A (Castennoitz based salts)

Ingredient	g l ⁻¹	ml ⁻¹
Nitrilo acetic acid	1.0	
CaSO ₄ .2H ₂ O	0.6	
MgSO ₄ .7H ₂ O	1.0	
NaCl	0.08	
KNO ₃	1.03	
NaNO ₃	6.89	
Na ₂ HPO ₄	1.11	
Deionised water		1000

Solution B

FeCl ₃ .6H ₂ O	28.0	
Deionised water		1000

Solution C (Nitchs Trace Element Solution)

H ₂ SO ₄	0.5	
MnSO ₄ .H ₂ O	220.0	
ZnSO ₄ .7H ₂ O	50.0	
CuSO ₄	1.6	
Na ₂ MoO ₄ .2H ₂ O	2.5	
CoCl ₂ .6H ₂ O	4.6	
Deionised water		1000

To 100 ml of solution A, add 1.0 g yeast extract (Difco) and 1.0 g of Bactotryptone (Difco). Add 10 ml of solution B and 10 ml of solution C and make up to 1000 ml with deionised water. Adjust the pH to 8.2 with 10 M NaOH and autoclave.

Table 7 Methylobacterium medium

Ingredients	g l ⁻¹	ml ⁻¹
CuSO ₄ . 5H ₂ O	0.005	
MnSO ₄ .4H ₂ O	0.01	
Sodium Molybdate	0.01	
Boric Acid	0.01	
ZnSO ₄ .7H ₂ O	0.07	
Cobalt Chloride 6H ₂ O	0.005	
Millipore Water		1000

2.4 INOCULUM FOR LABORATORY MODEL

The inoculum for the laboratory model was obtained from a corroded copper tube extracted from the Victoria Infirmary during the site survey in 1987 (page 73). A section of tube was identified for removal in the laboratory block where corrosion was

found to be severe. The water to this section was turned off and drained into a sterile jerrican before the section of copper tube was cut out using a hacksaw. Upon removal, one end was covered with a sterile endcap (sterilised using a portable autoclave, Prestige, UK Ltd.), before filling the tube with source water from the sterile jerrican and sealing the open end with another sterile endcap. The copper tube was transported back to the laboratory within 48 h and placed in the entry port of an anaerobic cabinet. A vacuum pump (Grant, UK) was used to evacuate the airlock to -25 in Hg. The airlock was gassed with oxygen free nitrogen (BOC) to -15 in Hg and the procedure repeated. The airlock was then evacuated to -25 in Hg before being gassed to zero in Hg with an anaerobic mixture of 80%Hydrogen:10% CO₂:10% N₂. Once inside the cabinet in the atmosphere of the anaerobic gas mix the copper tube was opened at one end and the contents placed in a sterile container. Using a sterile dental probe, scrapings of the inner surface of the copper tube were placed in Pages' amoebal saline (Pages, 1967) in sterile bijoux bottles before being stored at -70°C. Recovery of microorganisms appeared satisfactory when the stored inoculum was thawed and grown in the continuous culture vessel.

2.5 LABORATORY MODEL

The design of the laboratory model was adapted from a system described by Keevil *et al.* (1987). A glass desiccater vessel (Jencons Ltd, Leighton Buzzard) was used as the base that contained a 25 mm magnetic flea (BDH) to provide a propeller for vortex mixing. The base was filled with 500 ml of distilled water in 100 ml amounts and the side of the base marked at these volumes. A titanium fermenter top plate (CAMR Engineering Services) with 9 large ports (22 mm diameter) into which were placed silicone rubber bungs (Esco Rubber/Sterlin Ltd, Middlesex) which held oxygen (Type G2, 10 mm diameter, Uniprobe, Cardiff), redox and pH electrodes (Russell pH Ltd, Auchtermuchty, Scotland). The pH electrode was calibrated against standards of pH 4

and pH 7, before and after autoclaving before being placed into the vessel top plate. The Eh electrode was calibrated against quinhydrone (BDH, UK) at pH 7 (+290 mV) and pH 9 (+176 mV). A cork borer nearest the diameter of each electrode was chosen to bore a hole in the silicone rubber bung using glycerol as a lubricant and the bung then worked over the glass section of the pH and redox electrodes. For the oxygen electrode the silicone bung had to be forced down from the top of the electrode to prevent damage to electrode sensor. The electrodes and silicone bungs were then washed in distilled water to remove excess glycerol. Four smaller ports (10 mm diameter) held glass tubing (internal diameter of 4 mm) externally covered with a few centimetres of silicone tubing to ensure sealing before being placed into the ports to be used as media inlet, outlet, air outlet and glass RT-temperature sensor (Anglicon Systems) (Fig. 2.1). The media inlet was made of glass and contained a bulbous section surrounding the media dropper to prevent grow back of organisms back up the media line. In the bulbous section was another inlet tube through which air was passed. This inlet was preceded by a $0.2\ \mu$ air filter (Sartorius) to prevent ingress of bacteria through the air pump. An air filter was also placed on the air outlet. One of the large ports was also used to hold a glass tube which was used as a sample port. Silicone tubing was connected from the glass port to a titanium sample port, into which was inserted a universal container, which had an air filter (Sartorius), onto which a syringe could be fitted to draw through a sample of the culture. All ports had brass screw caps which were screwed down to give an air tight seal. A silicone rubber O-ring greased with silicone sealant was placed between the base and lid, before using two titanium outer rings which clamped the top plate and glass base together by tightening 6 oppositely facing screws (Fig. 2.2). All components were made of glass and titanium or silicone rubber to prevent leaching of nutrients or metals, from unsuitable components, that would otherwise alter the water chemistry.

When assembled, all but one inlet to the vessel was clamped off and the vessel was completely immersed in water in a sink unit to test the integrity of the seals by

pressurising the vessels to 10 lb. pressure. Before sterilisation the rubber seals covering the ports in the electrodes, which allow the electrolyte to be filled, were opened to prevent pressure build-up rupturing the membrane seals during autoclaving. All media inlet and outlet ports were clamped off and air filters taped up to maintain a vertical position, for if these filters bend towards a horizontal position there is the possibility that they will fill with moisture and block up. The resulting pressure build-up may damage the vessel and electrodes. Once the vessel was air tight it was placed in a metal container for protection, then sterilised by autoclaving at 121°C for 30 min and allowed to equilibrate overnight before removal from the autoclave.

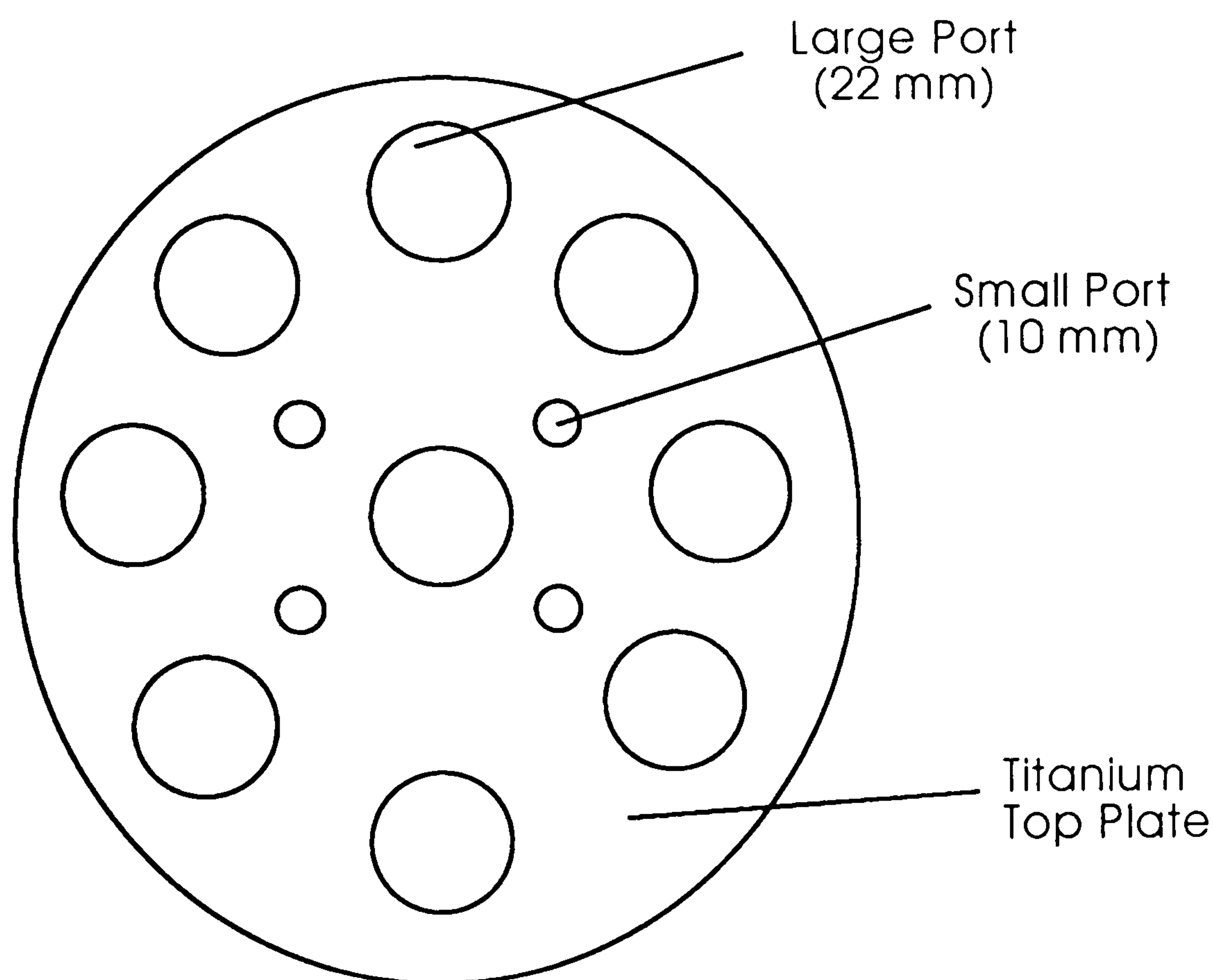


Figure. 2.1 Top plate of chemostat vessel.

After removal from the autoclave the vessel was again submerged in water to test the integrity of the seals and if this was satisfactory the vessel was set up as a chemostat. The electrodes in the vessel were connected to the microprocessor fermenter controller and the oxygen electrode calibrated to zero as the oxygen concentration would be low due to the recent autoclaving. This was carried out by making sure that the air inlet

and outlet filters were not blocked and passing oxygen free nitrogen into the vessel. With the oxygen meter set to polarographic the gain on the microprocessor fermenter controller was adjusted to read zero (approximately 30 min). Having set the zero scale, air was then blown into the vessel from the air pump in the controller and the slope adjusted to register 100%, (approximately 30 min). The pH and Eh electrodes were previously calibrated, as above, before being placed into the vessel to be autoclaved again.

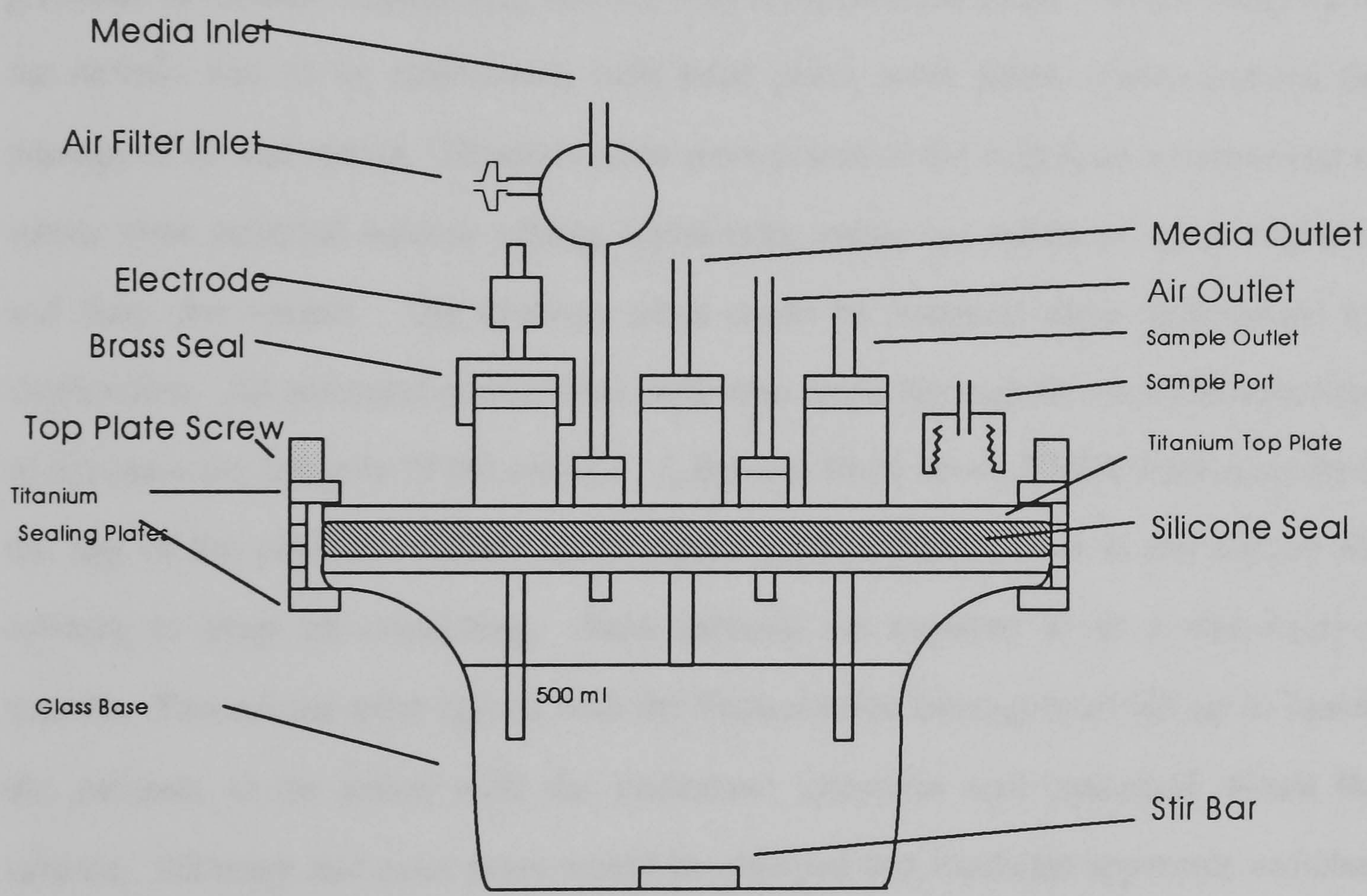


Figure 2.2 Schematic drawing of chemostat vessel

Two vessels were prepared as above with the media outlet of the first vessel connected to the media inlet of the second vessel to produce two continuous culture vessels in series. Each vessel was controlled by a microprocessor controller which was an integral unit containing temperature controller, air pump, Eh, pH, oxygen meters,

media pump and effluent pump. Air was continually pumped into each vessel. (0.5 l h^{-1}) and the oxygen concentration was controlled by the speed of the magnetic stirrer (LH Fermentation), positioned below each vessel. Otherwise the stir speed was set at 160 rpm. An asbestos mat (1.5 cm thick) was placed between the heating element (Anglicon) and the stirrer to protect the stirrer from heat.

The vessels were housed within a class III safety cabinet with a view to incorporating pathogenic microorganisms into the culture at a later date and therefore to provide operator protection (Fig. 2.3). These cabinets were designed with the assistance and guidance of CAMR Engineering section who manufactured them. As the integrity of the cabinet had to be maintained, bulk head plates were fitted, which enabled the passage of air and media. Titanium tubes were placed in the bulk head to either end of which were attached silicone tubing, transferring either air, media or spent media to and from the vessels. The titanium tubes could be removed when appropriate for sterilisation. All electrical connections were also made through an electrical bulk head to maintain the integrity of the cabinet. A fan was fitted above HEPA filters present in the top of the cabinet, to draw air in across another HEPA filter in the side of the cabinet, to keep air circulating. Such cabinets are required to be tested every 6 months. Procedures were agreed with the Fermentation Management Group to enable the cabinets to be tested with the chemostat apparatus still contained within the cabinet. All entry and exist ports would be clamped and electrical apparatus switched off before swabbing down equipment with 70 % isopropanol. Such procedures ensured that the equipment was out of use for a minimum period of time.

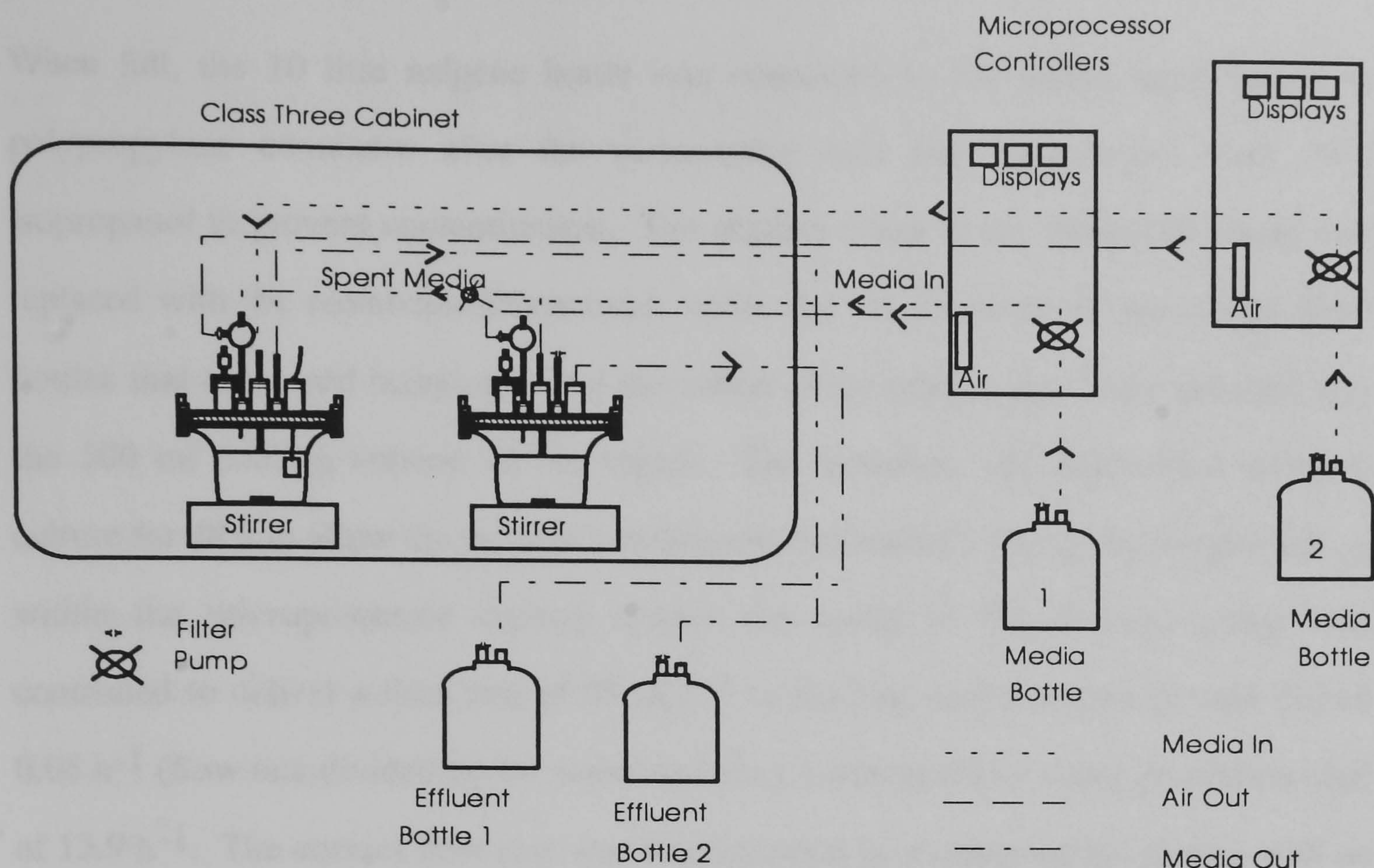


Figure 2.3 Layout of controller, chemostat vessels and cabinet

Growth medium for the continuous culture laboratory model was water obtained from the hydrant at the Victoria Infirmary. Four empty 20 litre jerricans were transported to Glasgow and returned full of soft water from the hospital. The water was placed at 5°C until required for use. After assembling the chemostat vessels in the class III cabinet, a 10 litre (high density polyethylene, Nalgene, UK) bottle was filled with soft water that was sterilised using membrane filtration (Colbourne *et al*, 1988b). A 147 mm filter housing of stainless steel (Sartorius, UK) was loaded with a 142 mm, 0.2 μm nylon membrane filters (Pall, Portsmouth) and sterilised at 121°C for 15 min. The housing was then connected to a sterile Nalgene bottle via a titanium sample port. The top of each Nalgene bottle was modified with three polyethylene connectors (media inlet, outlet and air filter) screwed through the top, such that an aseptic seal could be maintained. Soft water was sterilised by pumping (R600 pump, Watson Marlow, UK) through the filter housing, sample port and into the 10 litre bottle, using a relatively slow speed, otherwise pressure on the tubing often resulted in the tubing perishing.

When full, the 10 litre nalgene bottle was connected to the media input line by a polypropylene connector after the surrounding area had been wiped with 70% isopropanol to prevent contamination. The distilled water in the chemostat vessel was replaced with the membrane-filtered soft water and the contents of one of the bijoux bottles that contained scrapings from the inside of the copper pipe were emptied into the 500 ml holding volume of the vessel. The inoculum was maintained in batch culture for 48 h to allow the inoculum to become established. Using the integral set-up within the microprocessor control system the speed of the nutrient pump was controlled to deliver a flow rate of 25 ml h^{-1} to the first vessel, a dilution rate (D) of 0.05 h^{-1} (flow rate divided by the vessel volume), equivalent to a mean generation time of 13.9 h^{-1} . The correct flow rate was accomplished by placing a glass pipette with an air filter at the top in the media in-line before the pump. Media could then be drawn into the glass pipette using a syringe and the pump set by measuring how long it takes to deliver a known volume. An auxiliary pump (Type R101, Watson Marlow) was used to pump spent media and cells from the first vessel into the second. The second vessel was also supplied with sterile media, as above, but at a rate of 75 ml h^{-1} resulting in a total flow rate of 100 ml h^{-1} , a dilution rate of 0.2 h^{-1} and equivalent to a mean generation time of 3.5 h^{-1} . Using another auxiliary pump spent media was pumped from the second vessel to an effluent container (10 litre nalgene bottle). When the 10 litre container was 80% full it was replaced by another sterile effluent bottle. Contents of the partially full effluent bottle were then sterilised by the addition of chlorox resulting in a 10 % (v/v) final solution. After a contact time of 24 hours the contents were washed down a designated sink with copious amounts of water.

2.6 QUANTITATION OF MICROORGANISMS IN THE PLANKTONIC PHASE

Bacterial populations within the water phase were quantified by removing an aliquot from the water phase of the vessel using the sample port. Planktonic phase samples were extracted from the vessel at weekly intervals. From each aliquot, serial dilutions were prepared with sterile distilled water and 0.1 ml was used to inoculate R2A (section 2.9), BCYE and GVPC agar plates in duplicate. The agar plates were then incubated at 30°C for 7 days.

2.7 DEVELOPMENT AND QUANTITATION OF BIOFILMS

To obtain copper coupons on which to establish biofilms a copper tube (15 mm, thin walled, not blasted, IMI Yorkshire Copper Tube Ltd.) was machined into coupons of 1.0 cm x 1.0 cm, with a 1 mm hole at one end. Copper coupons were suspended from silicone bungs using copper wire (99%) . Glass coupons (Optiglass, UK) 1.0 x1.0 cm with a 1mm hole at one end, to be used as a control were suspended from silicone rubber bungs using titanium wire. Both sets of coupons were then rinsed in acetone to degrease the coupons and air dried by placing in medical flat bottles. The tops were covered with aluminium foil before autoclaving (Prestige laboratory autoclave). Each port hole area where the tile assembly was to be placed into the top plate was swabbed with 70% isopropanol before the sterile bung assembly was placed into the port hole on top of the fermenter head, such that coupons were suspended into the media in the vessel. The culture was established at 40°C and maintained at this temperature to allow coupons to be immersed in the vessel for 21 days. At intervals of 1, 4, 7, 14 and 21 days a glass and copper tile assembly was extracted and replaced by a sterile bung. The tile assembly was placed in a medical flat and transported to the microbiology

bench where each tile was removed from the wire, using sterile forceps, and gently rinsed in 10 ml sterile distilled water to remove unattached bacteria. Subsequently coupons were placed in 1.0 ml sterile distilled water and aseptically scraped and vortexed to remove adherent biofilm. Serial dilutions, in sterile distilled water, were prepared and a 0.1 ml aliquot of each dilution was then spread in duplicate onto R2A, BCYE and GVPC agar plates using plastic spreaders. Inoculated agar plates were then incubated for 7 days at 30°C.

2.7.1 Temperature Profile

When the last set of coupons was removed from the vessel the temperature was raised by 5°C and the culture allowed to equilibrate for 5 days before the immersion of another set of coupons. Increasing the temperature took approximately 20 min. Temperature of the culture in the vessel was increased after each investigation of biofouling at a particular temperature. Biofilms were generated from 40°C to 60°C and were monitored at 1, 7, 14 and 21 days with scanning electron microscopy of the sample surfaces. The temperature of the test vessel was increased up to 75°C and then decreased to 40°C before the biofouling was again monitored over the temperature range of 40-60°C. This was carried out to investigate whether exposure to temperatures above 60°C would have an effect on biofouling profiles. Biofilms generated during this latter experiment were monitored at 1, 7 and 14 days but without scanning electron microscopy of the samples surfaces.

2.7.2 Thermal Pasteurisation

2.7.2.1 Biofilm generation at 45°C

Copper coupons were prepared (as in section 2.7) and immersed for 42 days into an auxiliary third vessel maintained at 45°C which was placed in-line and down-stream from the second vessel. The coupons were then removed from the auxiliary vessel and placed into the second vessel where the temperature was maintained at 60°C and coupons removed over a 15 h period.

2.7.2.2 Biofilm generation at a range of temperatures

Copper and glass coupons were exposed to varying temperatures for 12 day periods starting with 50°C followed by 55°C then 60°C before the temperature was changed to 45°C. Copper and glass coupons were then removed for biofilm enumeration before the temperature was changed to 60°C with more coupons being removed after 1 and 2 h respectively.

2.7.3 Acid treatment of established biofilms

Biofilms were generated on copper coupons for 14 days then extracted aseptically and immersed in 10 % (w/v) citric acid (Sigma, UK) for up to 4 h. The viability of the culture was ascertained at 0.5 h, 1, 2 and 4 h. To study the recolonisation of citric acid treated coupons, biofilms were again generated for 14 days, treated with citric acid for 0.5 h and rinsed twice in sterile distilled water then re-immersed into the vessel. Copper coupons were removed and viability of the biofilm assessed as above. Comparison procedures were repeated using 5% sulphamic acid (Sigma, UK).

2.7.4 Increasing the calcium carbonate concentration of the soft water

Calcium carbonate (Sigma, UK) was added to the continually mixed nutrient bottle supplying vessel 2 resulting in a final concentration of 80 ppm in the continuous culture vessel. The normal concentration for this soft water was less than 20 ppm calcium carbonate.

2.7.5 Laboratory apparatus to simulate corrosion of copper

Two new vessels were assembled as in section 2.5 (page 51). Vessel 1 was supplied with soft water, stored at 5°C to minimise metabolic activity and chemical changes within the water. The water was not exposed to other treatments such as sterilisation. The reason for this was to supply this first vessel with the same soft water as was present in the water circuit of the institutional building where the copper tube failure was occurring. Temperature within the vessel was 40°C, with a flow rate of 70 ml h⁻¹ and the culture was stirred at 160 rpm. Effluent from this vessel was pumped through a 10 m copper tube coiled to occupy a drying cupboard which was maintained at 40°C. Upon leaving the copper tube, the water passed through a 0.2 µm capsule filter (Gelman Scientific, UK) such that sterile water without particulate matter was released into the second vessel (prepared as in section 2.5, page 56). As all the bacteria would also have been taken out by the filter the vessel was inoculated with a bacterial culture obtained from the first vessel. Copper coupons were prepared as in section 2.7 and immersed into both vessels such that a comparison could be obtained by the presence or absence of particulate matter and removed from the vessels at 12 months old. Microscopical examination of the copper coupons were compared between those immersed in the first vessel supplied with particulate matter and the second vessel downstream of the copper tube.

A section of copper tubing (25 cm) was removed from the 10 m copper tube using a plumbers cutter. The area was swabbed with 70% alcohol before and after removal. Endcaps were placed over one end and the tube filled with sterile distilled water before the other end was sealed with another endcap. The tube was then sealed in a nylon autoclavable bag and transported to the laboratory. After removing one of the endcaps, the tube was held in a vice while 2 cm were cut from one end, to remove area damaged by the endcaps. Two parallel incisions, 2 cm long, were cut horizontally along the tube and one vertical cut such that a section of copper tube could be removed. This method allows for a section of copper with the least amount of curvature to be removed which does not need to be flattened out for visualisation.

2.8 MONITORING OF THE WATER SUPPLY DURING THE SITE SURVEYS

A Hydrolab Surveyor II, supplied by Thames Water Utilities plc. was used to monitor the physical and chemical parameters (temperature and oxygen) of the hot water supply. This apparatus consisted of five separate sections: display unit, data cable, battery pack (13.9 V), field data logger and Sonde sample circulator (Fig. 2.0). The Sonde apparatus which housed the electrode probes was connected to the display unit via a data cable in a high pressure housing to measure temperature and dissolved oxygen (DO). The probes were calibrated at Thames Water before being taken out on site. A suitable area next to a water tap was chosen for the apparatus and the probe housing was immersed in the manually controlled water flow coming from this tap, with continual measurement of the parameters. Monitoring of the temperature and DO commenced at about 1700 h and continued until the following morning. The outlet of the cistern tanks was flushed for one minute before measuring the temperature. Five samples (approximately 100 ml) were manually decanted from the tap, immediately filtered and stored at 5°C for assimilable organic carbon (AOC) analysis. The AOC is

the carbon available to the microorganisms for growth and is indicated by ATP determination. The procedure is based upon the principle that the more AOC i.e. nutrients available to the bacteria then the more ATP will be produced with results expressed as units of ATP (equivalent to 10^{-10} g ATP l⁻¹) (Stanfield and Jago, 1987). Analysis of water chemistry and coliform counts were carried out at Thames Water Utilities Plc., New River Head, London.

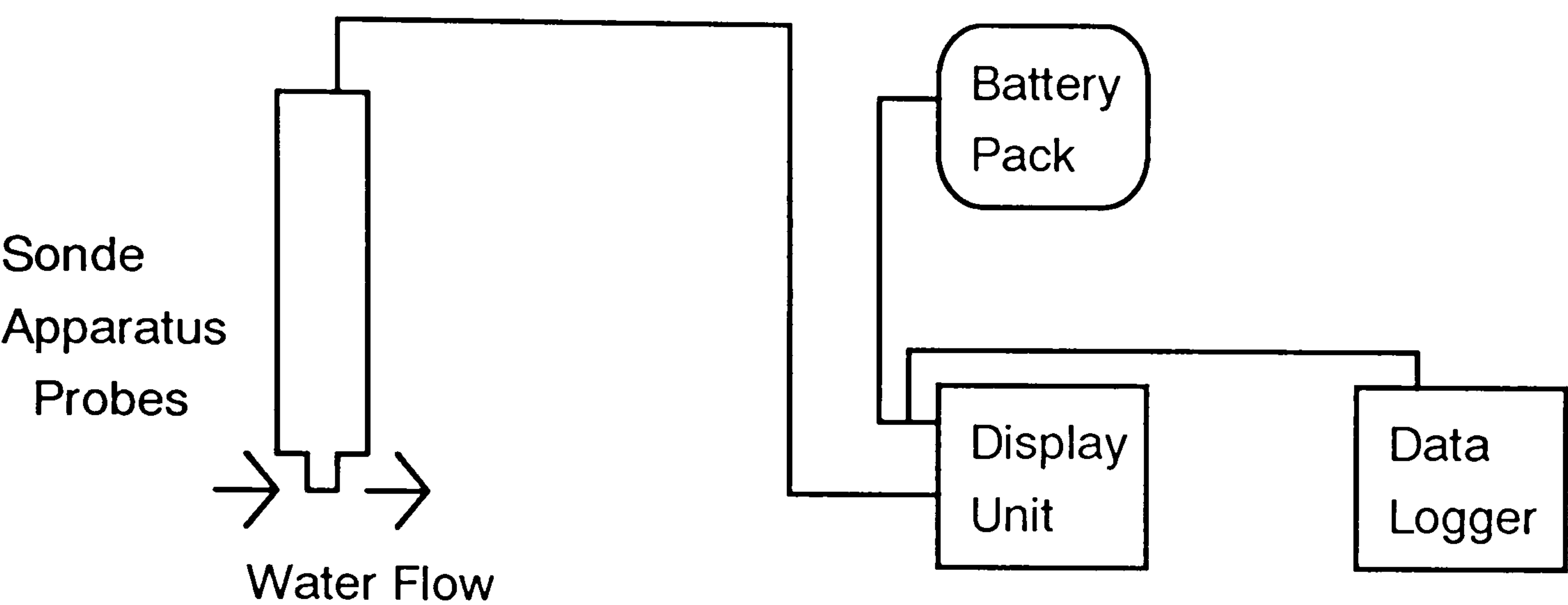


Fig. 2.0 Hydrolab surveyor II which was used to monitor the water parameters.

2.9 EXTRACTION OF COPPER PIPES

Approximately 0.5 m of copper tubing was selected for removal from the area of the building where monitoring was to be carried out. A 1 l sterile container was filled from the tap at the end of the circuit from which the section was to be remove`. The water was then turned off and, after draining, a section of copper tube was cut out. Visual inspection of the internal surface was carried out and the appearance was recorded with a 35 mm camera while the tube was held up to the light. After sealing the tube at one end with sterile endcaps, (sterilised using a portable automatic autoclave, Prestige, UK) the tube was filled with water from the 1 litre sterile container, sealed at the other end and stored at 5°C before being transported back to the laboratory.

2.10 ANALYSIS OF THE COPPER PIPING DURING THE SITE SURVEYS

In the laboratory, the copper pipes were placed in an anaerobic cabinet maintained with 80% N₂:10% H₂:10% CO₂, to prevent injury to any facultative or obligatory anaerobic bacteria present in the biofilm. A sample of the water phase from the pipe was used to quantify the planktonic bacteria and approximately 1 cm² of the surface was aseptically scraped for the detection of anaerobic bacteria in the biofilm. Aerobic diluents were also prepared for the detection of aerobic bacteria. Where appropriate particular tubercles were removed with 6M HCl, then rinsed in sterile distilled water before observing the surface for the characteristic pepper pot pitting. Sections of the copper tube were also taken for SEM analysis.

2.11 CHANGE FROM CONTINUOUS CULTURE TO BATCH CULTURE

Following the aseptic extraction of copper coupons from the vessel at 60°C the effluent from vessel one at 30°C was diverted such that it did not act as a seed for vessel 2 which was still supplied with sterile media. The number of planktonic bacteria (per ml) present in vessel two, in batch mode, was then monitored over a 5 day period.

2.12 MICROSCOPICAL EXAMINATION OF BIOFILMS AND PLANKTONIC POPULATIONS

Aliquots of 9 ml were aseptically extracted from each vessel, to which was added 1 ml of a 0.2 % stock solution of 2-(p-iodophenyl)-3-(-p-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Fluka Ltd, UK) in sterile distilled water, final concentration of 0.02%, and incubated at 30°C for 1 hour. After incubation the suspension was stained further

with a 1 ml aliquot of 0.2% stock solution of acridine orange (AO, Sigma, UK) (final working concentration of 0.02%), in sterile distilled water and after 2 minutes was filtered through a 47 mm, 0.2 μm polycarbonate membrane (Nuclepore, UK) using a buchner funnel and drawn through using a hand vacuum pump (Nalgene). Both stains were pre-filtered through a 0.2 μm filter (Sartorius) prior to use. The filter was then microscopically examined in the fluorescence mode to count bacterial cells whose DNA and RNA had stained with AO. The filter was also examined in transmission mode to count those metabolically active cells which had reduced the INT dye to a red insoluble dye, visible within the cell. Up to 10 fields were counted per mode to obtain average counts. An example of the calculation is shown below:

$$\text{Number of bacteria} = \text{number in field of view} \times \text{area of filter} / \text{area of field of view}$$

$$\text{Area of filter} = \pi r^2$$

$$\begin{aligned} \text{Diameter of working area of filter} &= 37 \text{ mm} \\ \text{i.e. radius} &= 18.5 \text{ mm} \\ &= 3.14 \times 18.5 \times 18.5 \\ &= 1075 \text{ sq. mm} \end{aligned}$$

$$\text{Area of field of view (x150)} = \pi r^2$$

$$\begin{aligned} \text{Diameter of field of view} &= 100 \mu\text{m} \\ \text{i.e. radius} &= 50 \mu\text{m} \\ &= 3.14 \times 50 \times 50 \\ &= 7853 \mu\text{m or } 0.00785 \text{ sq. mm} \end{aligned}$$

$$\begin{aligned} \text{Number of bacteria (per filter)} &= \text{average number in field of view} \times 1075 / 0.00785 \\ &= \text{average number in field of view} \times 136942 \end{aligned}$$

The result can then be calculated to obtain the number of bacteria per ml.

2.12.1 Conventional light microscopy (adapted)

Microscopical examination of the filters was carried out using a Nikon Labophot-2 which combines both epi-fluorescence and differential interference contrast (DIC) (Fig. 2.4). A B-2A filter block was used for the epi-fluorescence. This has a 510 nm wavelength dichroic mirror, an excitation filter of 450-490 nm wavelength and a barrier filter of 520 nm wavelength. An IGS (immuno-gold system) block was used for DIC. The objective lenses were non-contact M Plan Apo lenses of 40 (0.5 numerical aperture, NA), 100 (0.8 NA) and 150 times (0.95 NA). Light source was a 100 W halogen lamp. Neutral density filters were used where necessary to suppress high background fluorescence. Fig. 2.5 demonstrates the route of image processing of samples.

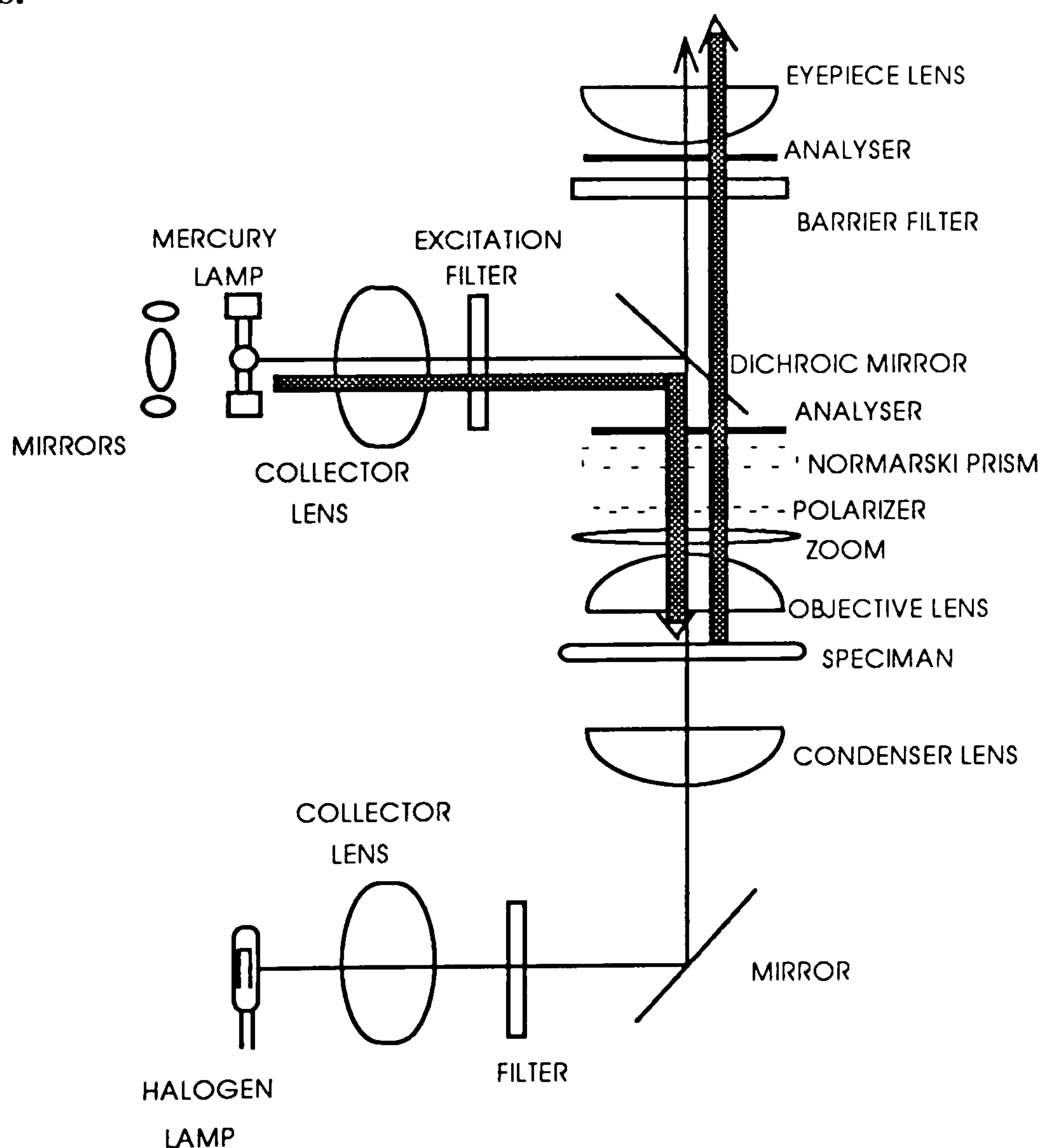


Figure 2.4 Schematic of adapted light microscope which consists of a conventional light microscope with episcopic UV fluorescence and DIC. Adaptations include; siting

of the polariser above the specimen (allowing opaque specimens to be viewed); enlarging the filter block housing to accommodate four filter blocks and mirror plates in the mercury lamp housing to increase the signal output.

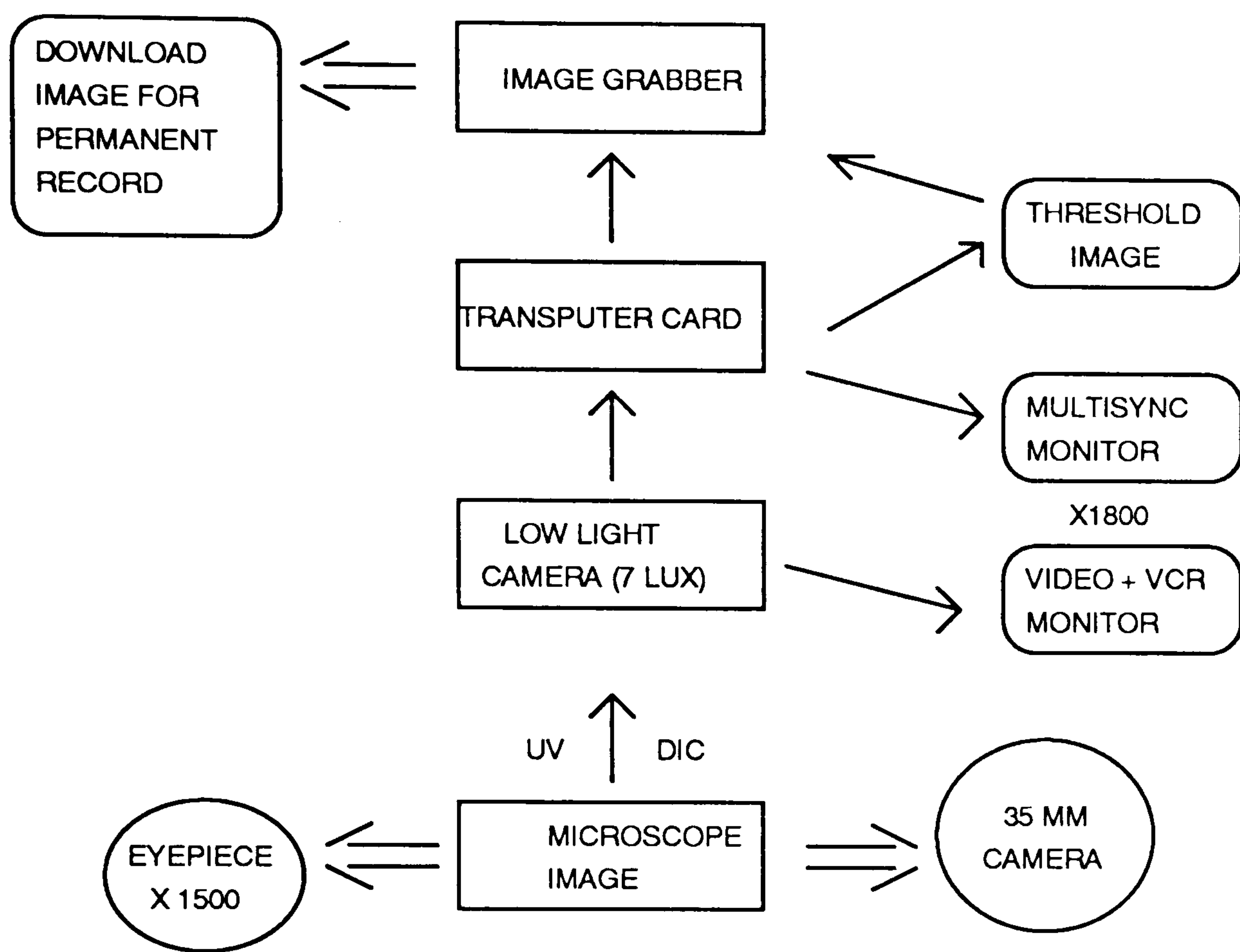


Figure 2.5 Process of imaging samples through the microscope.

2.12.2 Scanning Electron Microscopy (Traditional Preparation)

The glass and copper coupons were immersed into 30 % absolute alcohol immediately after being taken from the laboratory model. After 15 and 30 min they were placed into 50% and 70% absolute alcohol respectively. Following immersion in 90% absolute alcohol (45 min) the coupons were placed in osmium tetroxide (5%) (BDH) for two hours before being placed into a dessicator. After transportation to the SEM suite, the coupons were gold sputtered before being viewed in a Cambridge SEM. Sample sputtering and image processing were carried out at CAMR by A. B. Dowsett. As the magnification of each print was known, a square template was cut out, allowing the number of bacteria within each print and so on each tile to be calculated. At least

10 areas were enumerated to obtain a mean value which was multiplied by the magnification resulting in the number of bacteria per cm².

2.12.3 Scanning Electron Micrography (Cryogenic Freezing Unit)

Samples of the copper tube were sectioned and maintained hydrated in a petri dish. An XL40 with cryogenic unit (Philips) based at Thames Water Utilities plc., Reading was used to examine the samples from the copper tube under the supervision of Dr. K. Colquhoun. The copper tube samples were then individually attached to an SEM sample holder in the transporter pot using SEM adhesive. The nitrogen slushing chamber was evacuated and a slush prepared by allowing the nitrogen to solidify. When gas was admitted, the end cap was removed before immersing the sample into the nitrogen slush. After freezing, the sample was placed into the microscope observation chamber via the transporter pot and sputter coated for 2 min at 1 mA with gold in an argon plasma. The specimen was then viewed.

2.12.4 Environmental SEM (ESEM)

The ESEM based at the Medical School at the University of Manchester is a fully integrated general purpose microscope and the samples were investigated under the supervision of Dr. Nick Long. A four-stage differential pumping system in the electron-optical column allows the entire specimen chamber to be maintained at gas pressures higher than those permitted in conventional SEM. Chamber pressure was maintained steady by means of a fully automated electronic pressure servo system enabling pressures between 1-50 torr.

Within the chamber low energy secondary electrons from the beam impact on the specimen surface and are accelerated towards the detector electrode by a moderate

electric field. The secondary electron detector is based upon the principle of gas ionisation. Successive collisions in the ambient gas molecules liberate more free electrons, resulting in a proportional cascade of current within the gas phase, where positive ions serve to effectively neutralise the destructive buildup of excess electron charge at the specimen. Image analysis of the results was carried out as below.

Images captured via a colour video camera (JVC TK-1085E) were then relayed via a 0.4x wide field lens to the Microeye TC image analysis transputer card and software (Digithurst, Royston) operated under the Windows 3 (Microsoft) environment on a PC 386 compatible computer (Elonex, London) (Fig. 2.5). The photographed images were relayed to a multisync monitor (Taxan 775; capable of automatically switching between non-interlaced and interlaced modes when the appropriate signal is recognised) for image analysis. Each grabbed image was framed with an 8 cm x 7 cm box on the screen to allow for analysis of a constant area. A threshold limit was set up to allow the software to identify biofilm against copper and a field and object scan carried out.

2.12.5 Scanning Confocal Laser Microscopy

Two vessels were assembled as in section 2.5, page 51. Copper coupons were prepared as in section 2.7, page 58, and immersed into both vessels such that a comparison could be obtained from the presence or absence of particulate matter. The coupons were removed from the vessels at 12 months old. Coupons were prepared in duplicate. One set were removed from the bung assembly and placed on hydrated tissue in a sterile petridish which was then sealed with parafilm to prevent leakage of water. The second set remained on the tile assembly which was put into a universal and sealed with parafilm such that the coupons would remain moist.

2.12.5.1 Scanning confocal Laser microscopy procedures

An MRC-600 (Biorad, Ltd.) scanning confocal laser microscope, located in the Biology department of the University of Saskatchewan, Saskatoon, Canada was used to analyse the biofilm samples and was supervised by Keith Hanson. The MRC-600 was fitted with an argon laser, maximum emission at 488 nm and excitation at 514 nm wavelength, and was mounted in the upright position above an optical light microscope (Microphot SA, Nikon ltd). The images from two different fluorescent markers were imaged simultaneously using an excitation filter and dichroic (DC) mirror to direct the image to either photomultiplier (PMT) 1 or 2 (Fig. 2.6). These filters could be changed without disrupting the optical alignment of the microscope thus allowing imaging of a single field of view under different spectral conditions. Facilities included a z-axis stepping motor providing precise increments of 1/1000 revolutions or multiples, essential for accurate optical sectioning using the aplanal oil objective lenses. Images were relayed to a 486 processor in an IBM PC/AT compatible desktop computer where they were integrated and digitised with a Kalman true-running-averaging filter. To visualise the surface of the copper tube, fluorescein (0.1%) was used as a negative stain. Bacteria were stained with either FITC or RITC. For fluorescein, imaging optical filters with a 488 nm and 510 nm wavelength were used, while for rhodamine imaging the 514 nm and 580 nm wavelength beam splitter were used. As an indicator of pH, 5-(and-6)-carboxy-2',7'-dichlorofluorescein (Molecular Probes, USA), was used as the excitation and emission wavelengths decrease due to acidification and would therefore indicate metabolic activity within the biofilm. In this procedure RITC was used to stain protein on the bacterial cell wall to complement the corresponding image of the pH indicator. The images were not subjected to any form of restoration e.g. nearest neighbour, delinear or non-linear deblurring methods.

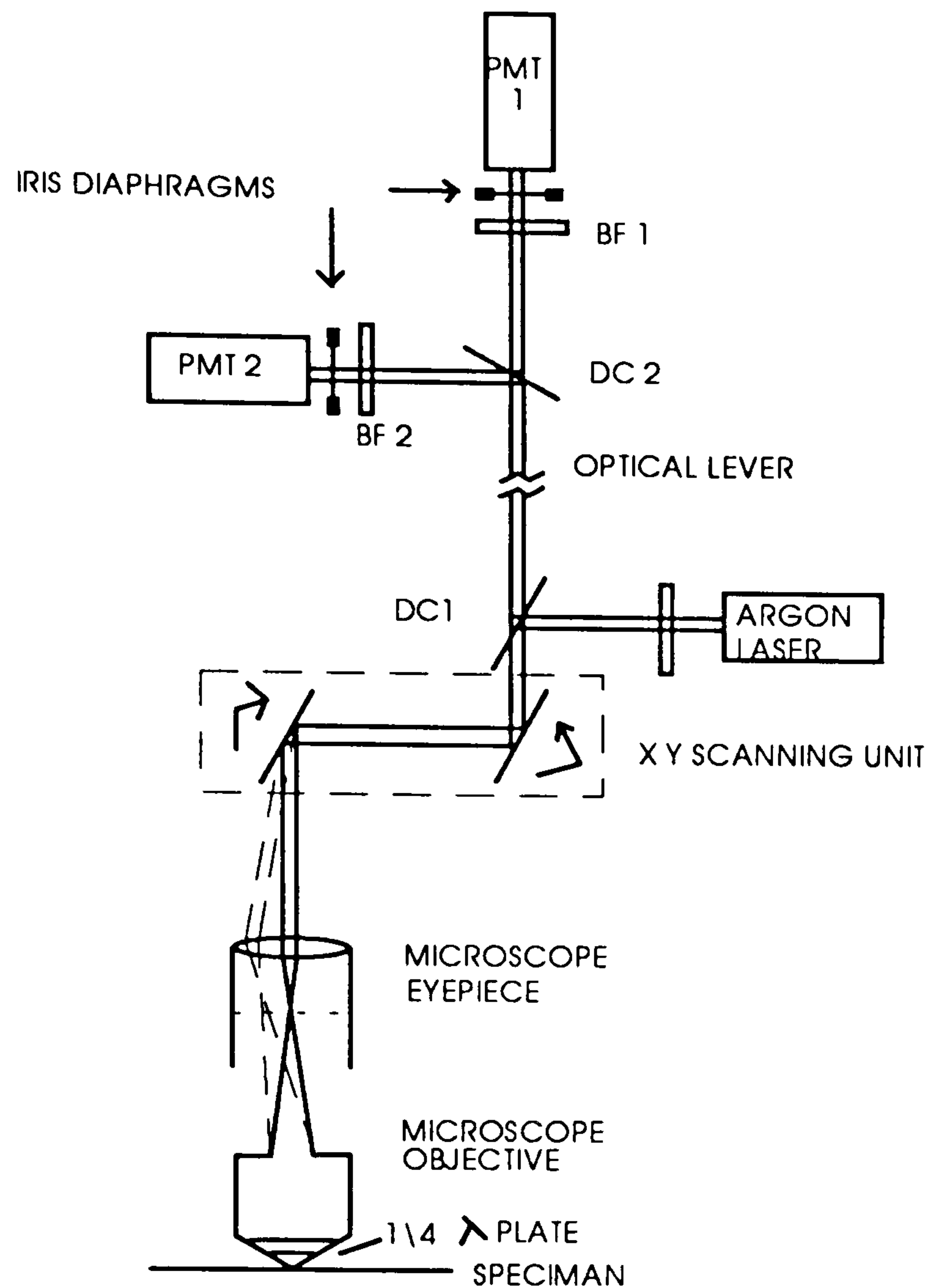


Figure 2.6 Simplified diagram of the confocal laser light path exhibiting both photomultiplier tubes PMT and dichroic mirrors (DC).

2.13 STATISTICAL ANALYSIS

All standard plate counts were carried out in duplicate and the mean reported. Biofilm counts were enumerated from one tile, however, periodically three coupons of the same material were analysed to ascertain the reproducibility of the technique. In all cases the errors were found to be not greater than 10% of the mean value.

The following criteria were met in order for the Wilcoxon Signed Rank Test to be used and so other tests were excluded.

- a) Looking at a difference between two experimental conditions.
- b) Each subject in one condition matched with a subject in the other condition.
- c) There is an interval scale of measurement.

d) Data for the two groups were not normally distributed.

The Wilcoxon Signed Rank Test has been utilised for testing for significance of difference between two samples which are related. For this test each subject or pair are subtracted from the other, giving a d value which is then ranked. The ranks are then summed resulting in a T value. With the use of statistical tables the T value can be looked up to obtain the probability (p value) and if the T value is less than or equal to the value given in the table then it is significant at that particular level.

CHAPTER 3

HOSPITAL SURVEYS

3.1 INTRODUCTION

An unusual form of corrosion in copper pipes in domestic water circuits has occurred since the 1980's, resulting in pitting and perforation of copper tube in such diverse areas as Scotland, Saudi Arabia and West Germany. This unique problem could not be explained simply in terms of chemical corrosion per se and suspicion has fallen on the possible involvement of microorganisms. In central Scotland the water systems of three hospitals had been identified as being affected by this unique form of corrosion. The Glasgow Evening Times (18 June 1987) reported that corroded pipes in two of the hospitals may cost as much as £3 million pounds to replace. Moreover, the escape of aerosolised water sprays through perforated tubing could pose a potential health risk, particularly if *Legionella* spp. are present in the water or able to grow in biofilms (Colbourne & Dennis, 1988; Keevil *et al.* 1988) adjacent to the perforations. Even in the absence of such pathogens, aerosolised aquatic bacteria may be involved in processes giving rise to humidifier fever and other lung infections. Growth of individual bacterial species form complex microbial consortia and the development of biofilms is markedly affected by the local environment, including changes in nutrient availability, pH, temperature, oxygen concentration, redox potential and metals concentration (Ellwood *et al.* 1982; Keevil *et al.* 1987, 1988; Glenister *et al.* 1988). Biofilm which is resistant to the toxic effects of copper may well harbour and protect potential pathogens which are normally inhibited by this metal (Burns *et al.* 1967; Gadd, 1992; Bitton and Feihofer, 1978; States *et al.* 1985).

Initially it was suggested that the copper corrosion was caused by the water supply but this was refuted by the water companies who suggested that the problem was due to tubes provided by the copper pipe fabricators. In view of the economics of replacing copper piping, estimated at £3M, the problem had to be investigated and it was the copper tube manufacturers who addressed this particular enigma of pepper pot-pitting of copper tube. In 1987 they commissioned a survey with the remit to investigate the

water supply, condition of the copper pipe work and to obtain an inoculum with which to initiate the laboratory model (Keevil *et al.* 1987). Following discussions with the appropriate authority (Scottish Office for Health) access was gained to two hospitals in 1987, the Victoria Infirmary and the Glasgow Royal Maternity, where this unusual form of corrosion was occurring, primarily in the hot water circuit. The strategy of the site survey was to investigate the water chemistry and microbiology of the water, at the inlet and at various locations in the hospital through to the faucets of the hot water supply. The temperature and dissolved oxygen profiles of the hot water were monitored continuously overnight with periodic samples taken to assess the assimilable organic carbon (AOC). AOC is one of the limiting factors for heterotrophic bacterial growth and so is at least in part responsible for bacterial proliferation (Ribas *et al.* 1991; van der Kooij *et al.* 1982; Stanfield and Jago, 1987). Copper tubing was also removed to be examined for the presence of microorganisms and corrosion on the internal surface. The inoculum for the laboratory model was obtained from the Victoria Infirmary and water from this particular hospital was also utilised in the laboratory model to simulate the environmental conditions under which the copper tube failures were occurring.

In February 1989, the Victoria Infirmary was revisited along with the Inverclyde Hospital in Greenock, Scotland where this form of corrosion had also been observed. As a comparison, in February 1989, visits were arranged to two institutional buildings on the East coast of Scotland where the problem had not been reported, the Eastern General, Edinburgh and the Stratheden Hospital, near Cupar in Fife. The main objective of this survey was to monitor the water parameters overnight for changes in the temperature, dissolved oxygen and assimilable organic carbon as well as to carry out microbiological analysis of the water and pipe surface.

3.2 MATERIAL AND METHODS

In each of the hospitals, the water supply parameters such as temperature and dissolved oxygen concentration were monitored over night, with water samples taken for assimilable organic carbon, chemical and microbiological analysis (standard plate counts) of the water at various locations in the buildings as detailed in sections 2.8 and 2.9 (pages 62 and 63). Sections of copper pipes were identified for removal for microbiological analysis (standard plate counts) and visualisation of corrosion using light and scanning electron microscopy as detailed in section 2.12 (page 67).

3.3 RESULTS

3.3.1 First site survey, 1987

3.3.1.1 Site A Victoria Infirmary

This hospital site was supplied with mains water from Loch Catrine in the Trossachs area, north of Glasgow. The continuous monitoring equipment was positioned in the outpatients department which received hot water via a recirculating loop from a calorifier maintained at 45°C. This calorifier was supplied with water at 9.9°C and pH 9.2 from a storage cistern which was in a very poor condition due to the absence of a cover. It had a storage capacity of 3000 gallons with a surface area of 130 square feet. A plank of wood had been placed along the top of the tank to allow passage across it and the paint on the ceiling above the tank was observed to be flaking and peeling.

(i) Continuous monitoring of the temperature, oxygen and AOC of the hot water supply

The incoming water supplying the cistern tank had an AOC of 7.4 (ATP $\times 10^{-10}$ g l⁻¹, defined as units of ATP) but in the tank itself the AOC was recorded as 12.4 units of ATP. The amount recovered from three different taps downstream of the calorifier varied between 4.8-7.8 units of ATP. When the temperature was monitored overnight it was initially at 52°C and fluctuated between 38-58°C until 20.30 h and 38-41°C thereafter until 07.00 h when it increased to 53°C (Fig. 3.0). The dissolved oxygen was between 7-9 mg l⁻¹ up to 19.00 h after which, less than 0.5 mg l⁻¹ was recorded. At 06.30 h the DO increased back to 8 mg l⁻¹.

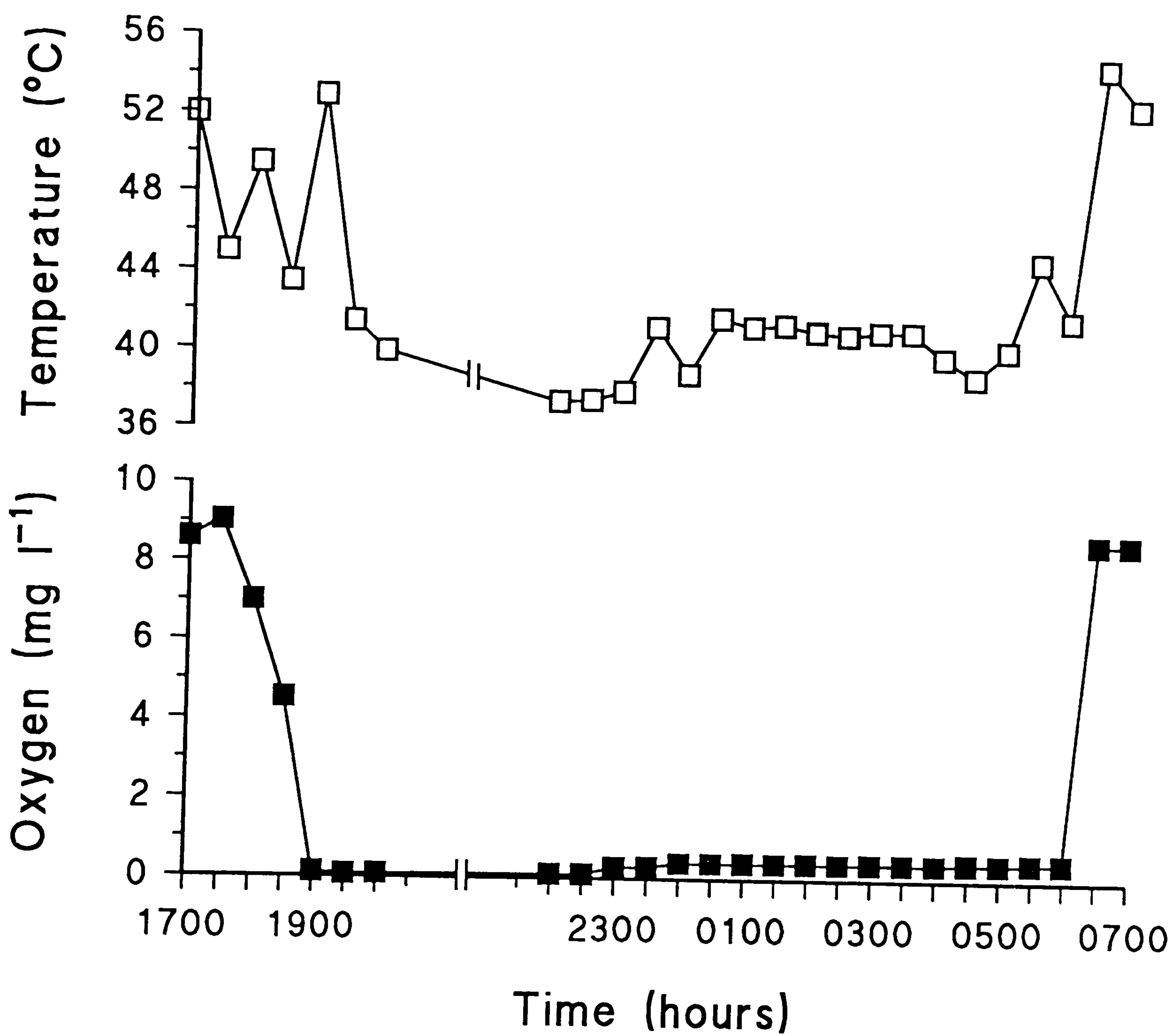


Figure 3.0 Continuous overnight monitoring of the temperature and dissolved oxygen profile during the first site-survey at the Victoria hospital in 1987.

(ii) Internal examination of the copper tubing

Copper tubing (approx. 0.5 m), approximately 100 m from the calorifier, was removed from the hot water circuit of the Outpatients department. Externally the tubing was covered in copper corrosion deposits and the lagging when removed was wet indicating that perforations had occurred in this section. Internally a large number of tubercles were present on the surface (Fig. 3.1). The water from inside this pipe contained black deposits.

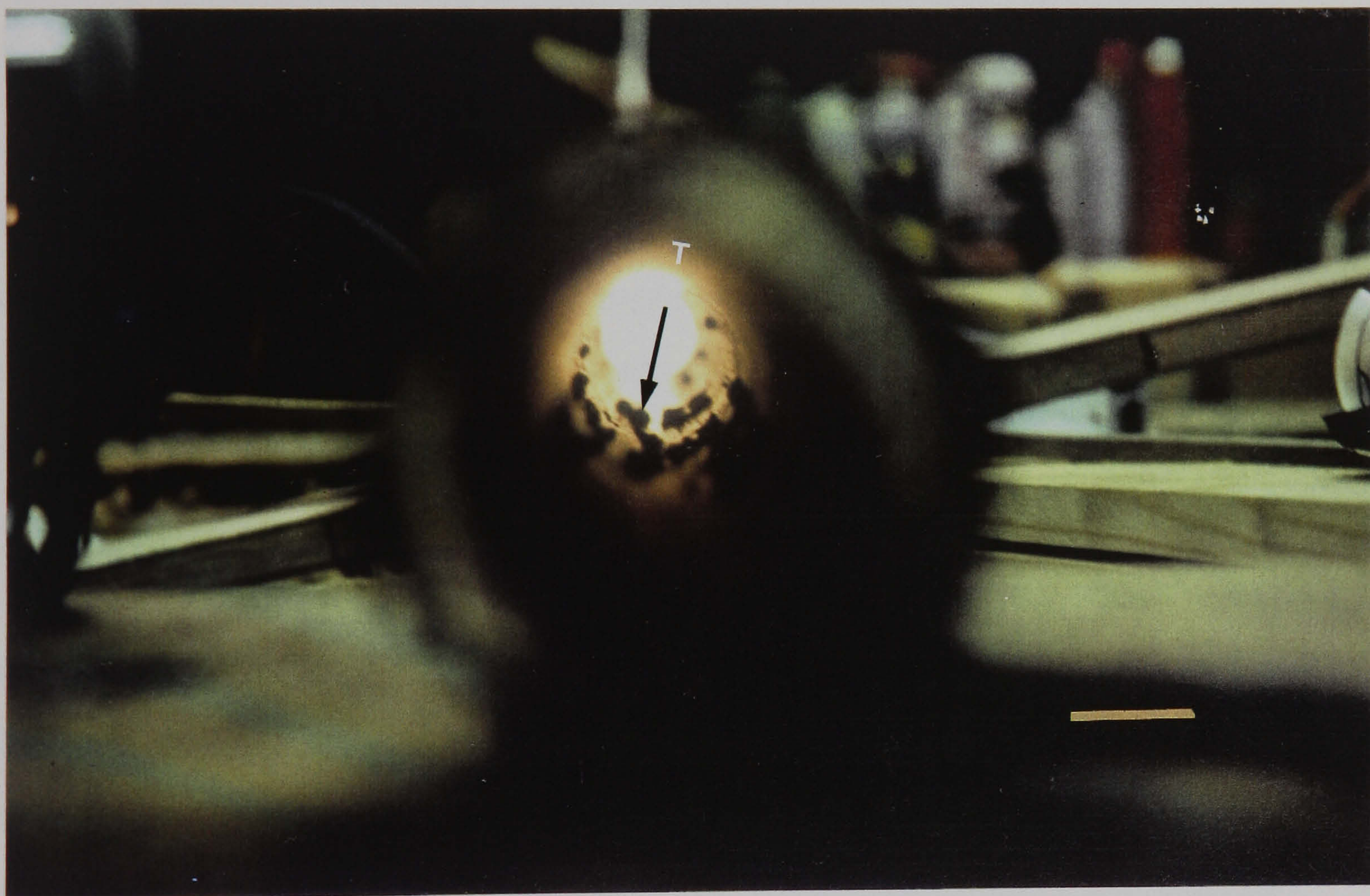


Figure 3.1 View of the internal area of the copper tube removed from the Victoria hospital outpatients department during the first site survey in 1987 (Marker bar denotes 10 mm and T denotes the position of the tubercles).

(iii) *Microbiological analysis of the water and copper tube surfaces*

The general microbiological condition of the source water was bacteriologically satisfactory with only 280 cfu ml⁻¹ recovered from the water which was at 9.9°C when it entered the building (Table 3.0). The temperature of the source water was stored at 10°C in the cistern tank where the number of bacteria recovered by standard plate count procedure was 500 cfu ml⁻¹ with only 1.0 x 10³ cfu ml⁻¹ recovered from the first hot tap from the calorifier. However, 5.0 x 10⁷ cfu per swab and 1.0 x 10⁷ cfu per swab, respectively, were recovered from one of the walls and a copper ballcock in the holding tank (Table 3.1, page 83). From the tap in the nurses home where the monitoring apparatus was positioned only 1.0 x 10³ cfu ml⁻¹ were recovered from a water sample however 5.0 x 10⁷ cfu per swab were recovered from the tap surface. Although fungi were only recovered from the tank, sulphate-reducing bacteria were recovered from all the cistern and hot water samples tested. Chemical analysis (Table 3.2, page 84) was carried out on water samples from several different sites, such as the first hydrant coming into the building, cistern tank, first tap off the calorifier and two other hot taps down stream of the water circuit. The colour of the water entering the building was slightly brown at a value of 13 Hazen units and remained so throughout the water system. Initially the pH was 9.3 but decreased at each site down to 7.6. Similarly the amount of solids present decreased at each sample site from 81 mg l⁻¹ to 37 mg l⁻¹. All other chemical parameters remained relatively constant apart from iron which increased from 64 to 332 µg l⁻¹, copper from 10 to 332 µg l⁻¹ and zinc from 12 to 25 µg l⁻¹. Visualisation of the surface of the copper pipe by SEM demonstrated that the thick slimy layer on the surface was composed of biofilm material (Fig. 3.2). Organisms recovered from the surfaces included *Methylobacterium* spp., *Alcaligenes* spp., *Flavobacterium* spp. and *Pseudomonas* spp., fungi and sulphate-reducing bacteria.

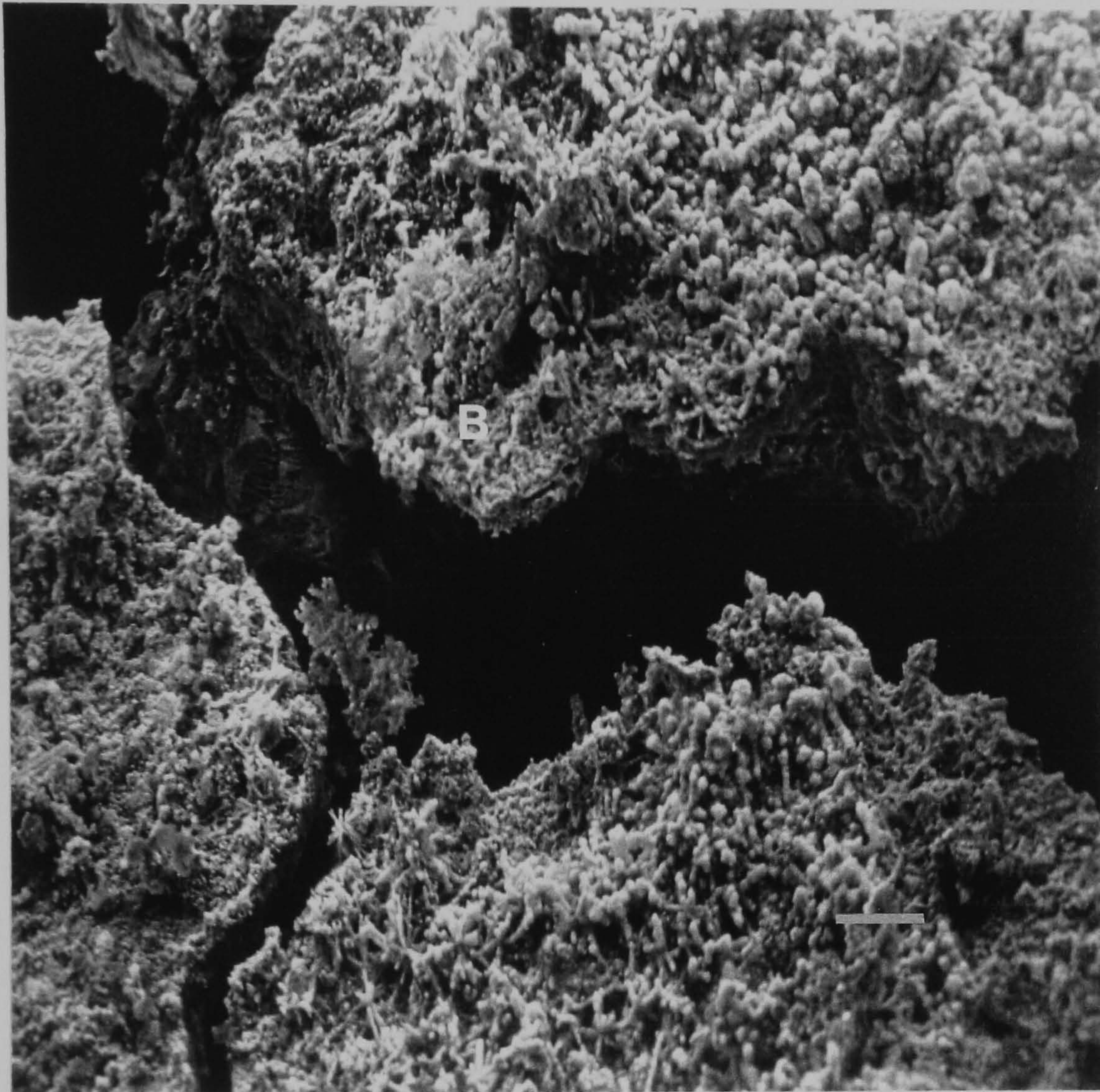


Figure 3.2 SEM of the surface of the copper pipe removed from the Victoria Infirmary during the first site survey in 1987 (Marker bar denotes $10\ \mu\text{m}$ and B denotes the biofilm layer).

Table. 3.0 Microbiological analysis of the sample sites during the first site-survey at the Victoria Hospital.

Site A I Victoria Infirmary						
	Hydrant	Tank	Tank Sediment	1st tap	Site 1	Site 2
Gram negative spp. ¹	280	500	nt	1000	1000	80
<i>Legionella</i> spp. ¹	0	0	0	0	0	0
Fungi ²	2	7	2	0	0	0
SRB ²	1	6	0	10	6	5
AOC ³	7.4	12.4	nt	4.8	7.8	5

No coliforms or *Pseudomonas aeruginosa* were detectable. ¹ denotes cfu ml⁻¹; ² denotes cfu per 100 ml and ³denotes units of ATP: 1st tap (of calorifier): Site 1 was a sample obtained from the hot tap obtained from the outpatients department and site 2 was a sample taken from a hot tap in the nurses home: nt denotes not tested.

Table 3.1 Comparison of the number of bacteria recovered from swabs and water samples from various sites at the Victoria Infirmary, Glasgow.

Sample	Temp (°C)	swab (x 10 ⁷ cfu)	ml ⁻¹ (x 10 ³ cfu)	OGN (%)	Ps.spp (%)	M. spp. (%)
Hydrant	10	nt	0.2	40	40	40
Holding tank	10	nt	0.5	40	40	20
Holding tank (wall)	nt	5.0	nt	40	40	20
Holding tank (Cu ballcock)	nt	1.0	nt	70	20	10
Tap (nurses home)	17-48	5.0	nt	40	40	20
Tap (nurses home)	17-48	nt	1	0	50	50

nt denotes not tested. OGN denotes Other Gram-negative bacteria; Ps. spp. denotes *Pseudomonas spp.*; M.spp. denotes *Methylobacterium spp.*

Table 3.2 Results of the chemical analysis of water sampled at the Victoria Infirmary.

Victoria Infirmary	S1	S2	S3	S4	S5
Temperature (initial)	-	10	23	28	17
Temperature (after flush)	10	-	51	43	48
pH	9.3	8.6	7.8	7.6	7.6
Colour, Hazen units	13	13	13	13	13

<u>mg l⁻¹</u>					
Solids	81(81)a	44(44)	50(50)	46(46)	37(37)
Total Organic Carbon (as C)	1.6	1.6	1.6	1.6	1.6
Ammoniacal Nitrogen (as N)	<0.2	0.2	0.2	<0.2	0.2
Carbon Dioxide	0.1	0.1	0.1	0.2	0.2
Total Oxid. Nitrogen (as N)	<0.2	0.2	0.2	0.2	<0.2
Chloride (as Cl)	<5.0	5.0	5.0	<5.0	5.0
Sulphate (as SO ₄)	3.0	4.0	4.0	3.0	3.0
Sulphide	<0.003	<0.003	<0.003	<0.003	<0.003
KMnO ₄ oxidisability	2.4	2.6	2.3	1.7	2.4
Reactive Phosphorous (as P)	<0.1	<0.1	<0.10	<0.1	<0.1
Total Hardness as (CaCO ₃)	<20	<20	<20	<20	<20
Calcium	5.0	4.0	4.0	3.0	3.0
Magnesium	0.60	0.7	0.6	0.6	0.60
Sodium	3.3	3.3	3.3	3.1	3.3
Potassium	0.3	0.3	0.3	0.3	0.3
Dissolved Oxygen	11.1	11.3	12.6	11.7	12.3

$\mu\text{g l}^{-1}$

Aluminium, Total	33	35	33	39	48
Iron	64(25)	74(35)	139(46)	111(63)	332(67)
Lead	<5.0	<5.0	<5.0	<5.0	<5.0
Manganese, Total	10(<10)	<10	<10	<10	24(<10)
Copper	10(10)	10(10)	139(46)	111(63)	332(67)
Zinc	12(10)	20(12)	10(10)	16(13)	25(13)

Data are expressed as total dissolved plus insoluble concentrations in parentheses. S1 denotes hydrant supply; S2 denotes storage tank; S3 denotes 1st tap off calorifier; S4 denotes hot tap, site one; S5 denotes hot tap, site two; Oxid. denotes oxidised.

3.3.1.2 Site B Glasgow Royal Maternity

This hospital site also received its water supply from Loch Catrine. The water in the cisterns, which had a sealed cover and were relatively clean, was found to be 15°C even though the source water was at 10°C. Two weeks prior to the visit the tank had been cleaned, bitumen painted and chlorinated before re-installing. Water from the first tap off the cistern was measured at 26°C prior to flushing. The cistern tank supplied a vertical calorifier which was thermostatically controlled at 50°C. Water from the base of the calorifier was measured at 27°C, but at the outlet to the hot water circuit the water was measured at 56°C. Sulphate-reducing bacteria and *Legionella bozmanii* were recovered from the unflushed drain; however, after flushing, neither of the above bacteria were recovered.

(i) Continuous monitoring of the temperature and oxygen of the hot water supply

Being from the same source, Loch Catrine, the chemical quality of the water was very similar to that received by the Victoria Infirmary (data not shown). There was no overnight monitoring of the temperature, dissolved oxygen or AOC water at this site.

(ii) Internal examination of the copper tubing

The copper tubing chosen for removal was on the 4th floor of the paediatrics department. The tubing was found to be externally clean and tidy with no signs of corrosion and was reported to have been in service for approximately 30 years. Visual examination showed that there were a few tubercles and only a thin layer of material deposited on the inner surface. (Fig. 3.3). SEM results indicated a relatively thin layer of material present on the surface (Fig. 3.4).

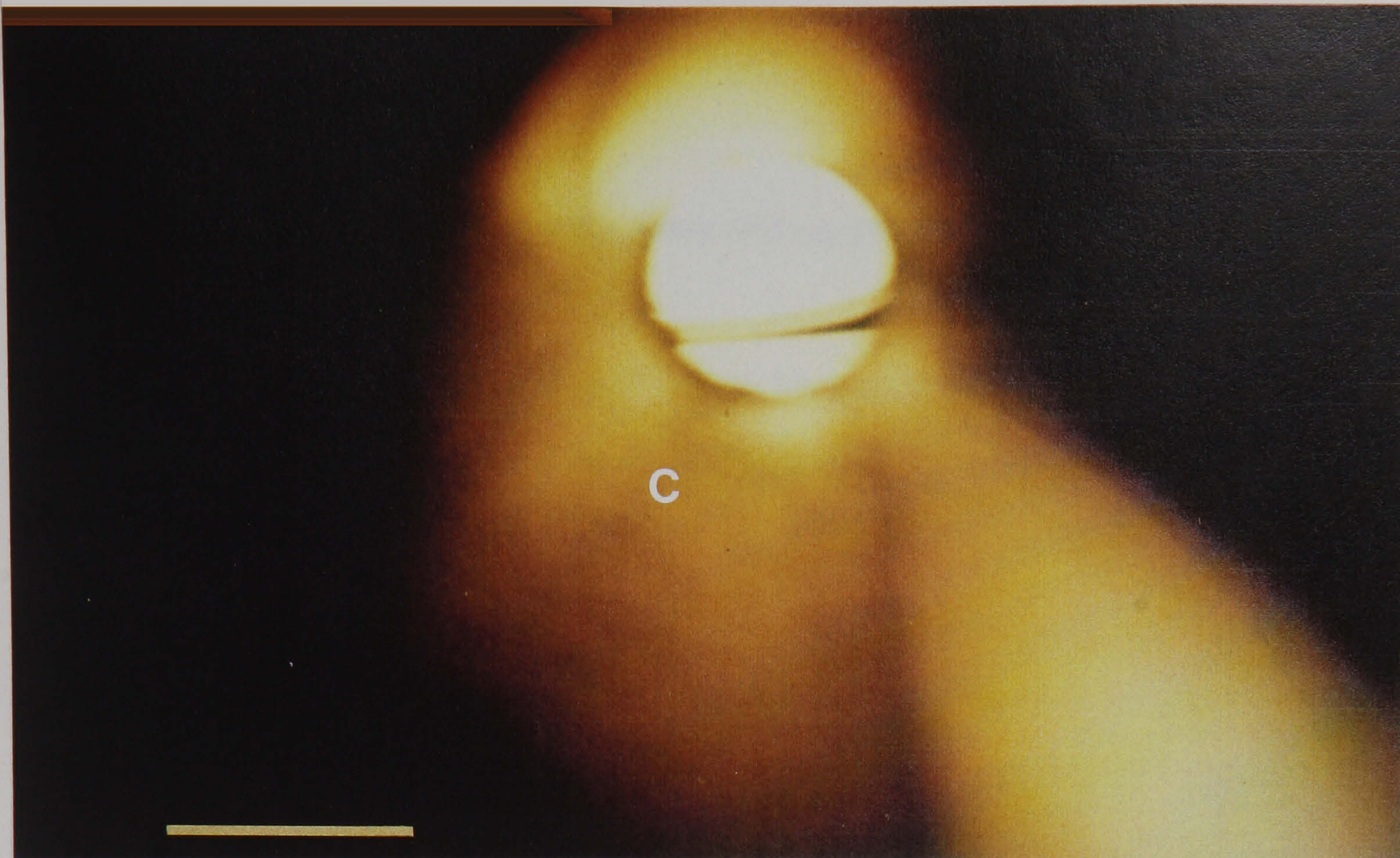


Figure. 3.3 Visualisation of the internal surface of copper tube samples removed from the Glasgow Royal Maternity (Marker bar denotes 10 mm and C denotes the copper surface).



Figure. 3.4 SEM of the surface of the copper tube removed from the Glasgow Royal Maternity (Marker bar denotes 10 μm ; C denotes the copper surface and B denotes the biofilm layer).

(iii) Microbiological analysis of the water and copper tube surfaces.

The water examined from the Glasgow Royal Maternity was also bacteriologically satisfactory with no coliform indicators being detected. The number of bacteria recovered from a water sample from the tank was only 50 cfu ml⁻¹ (Table 3.3), however 1.0 x 10⁸ cfu per swab (Table 3.4) were recovered from the tank wall. Samples taken immediately when the drain valve of the calorifier was opened contained 3.6 x 10⁶ cfu ml⁻¹ and the temperature was 27°C, however, after the tap was flushed for approximately 1 min it had risen to 56°C with only 1.0 x 10³ cfu ml⁻¹ of bacteria being recovered. From the first hot tap after the calorifier, the temperature was 59°C and only 2.0 x 10³ cfu ml⁻¹ were recovered from the water sample from this tap, however 2.5 x 10⁷ cfu per swab was recovered from this tap. The water from the tap furthest from the calorifier in the paediatrics department was recorded at 56°C with only 1.0 x 10³ cfu ml⁻¹ being recovered from the water sample but 5.0 x 10⁷ cfu per swab from the tap. Although fungi were present in the cistern and calorifier they were not detected down-stream. The number of aerotolerant microorganisms in the vertical calorifier was only 1.0 x 10³ cfu ml⁻¹ with 600 cfu ml⁻¹ of *Legionella* spp. recovered. No SRB were detected in any of the samples from this site.

Table 3.3 Microbiological analysis of the sample sites during the first site-survey at the Glasgow Royal Maternity.

Site B Glasgow Royal Maternity					
	Hydrant	1st tap off tank	Vertical calorifier	1st Tap	Site1 off cal.
Gram-negative spp. ¹	50	50	1000	2000	1000
<i>Legionella</i> spp. ¹	0	0	600	0	0
Fungi ²	2	12	3	0	0
SRB ²	0	0	0	0	0
AOC ³	8.1	3.4	nt	1.4	2.2

No coliforms or *Pseudomonas aeruginosa* were detectable. ¹ denotes cfu ml⁻¹ and ² denotes cfu 100 ml⁻¹. ³ denotes units of ATP. 2nd hot tap denotes the tap furthest from the calorifier which was on the 3 rd floor in the paediatrics block: nt denotes not tested.

Table 3.4 Comparison of the number of bacteria recovered from swabs and water samples from various sites at the Glasgow Royal Maternity.

Sample	Temp(°C)	swab (x 10 ³ cfu)	ml ⁻¹ (x 10 ³ cfu)	OGN (%)	Ps. spp. (%)	M. spp. (%)
Holding tank(wall)	15	10	nt	50	30	20
Vertical cal(before flush)	27	nt	3600	75	25	0
(after flush)	59	nt	1	40	40	20
First Hot Tap (after cal)	59	2.5	nt	0	100	0
	59	nt	2	90	10	0
Tap furthest from calorifier	56	5.0	nt	90	10	0
	56	nt	1	100	0	0

nt denotes not tested

3.3.2 Second Site Survey, February 1989

3.3.2.1 Site A, Victoria Infirmary

This hospital was investigated in 1987 (Section 3.2) and was also studied during the second survey in 1989 because the pepper pot-pitting corrosion was still occurring. The laboratory block was chosen as the building in which to operate the monitoring apparatus as this was where copper tube failures were still being reported since the original visit in 1987. The hot water supply came from a calorifier fed by a cistern on the roof of the main hospital. Although the cisterns had been renovated since 1987, they were observed to be in a rather poor condition with badly fitting lids. The cisterns fed a single coal-fired calorifier which dispensed hot water to the whole hospital. As mixer valves were not used on water outlets, the hot water supplied to faucets was maintained at 43°C, as this temperature would minimise scalding of hospital staff and patients.

(1) Continuous monitoring of the temperature, oxygen and assimilable organic carbon (AOC) of the hot water supply.

Monitoring of the temperature, oxygen and AOC (reported as units of ATP) commenced at 17.30 h at a faucet in an office of the laboratory building. Initially the water temperature was 43°C but decreased to 33°C by 20.00 h and then fluctuated between 27-37°C until 05.00 h the following morning before increasing to 45°C at 05.30 h (Fig. 3.5). The water supplying this faucet was found to be highly aerated, containing between 8 - 10 mg l⁻¹ of dissolved oxygen. Although the incoming water was found to contain 4.0 units of ATP at 17.30 h this decreased to 1.5 units of ATP at 18.00 h and continued to decrease for the remainder of the monitoring period.

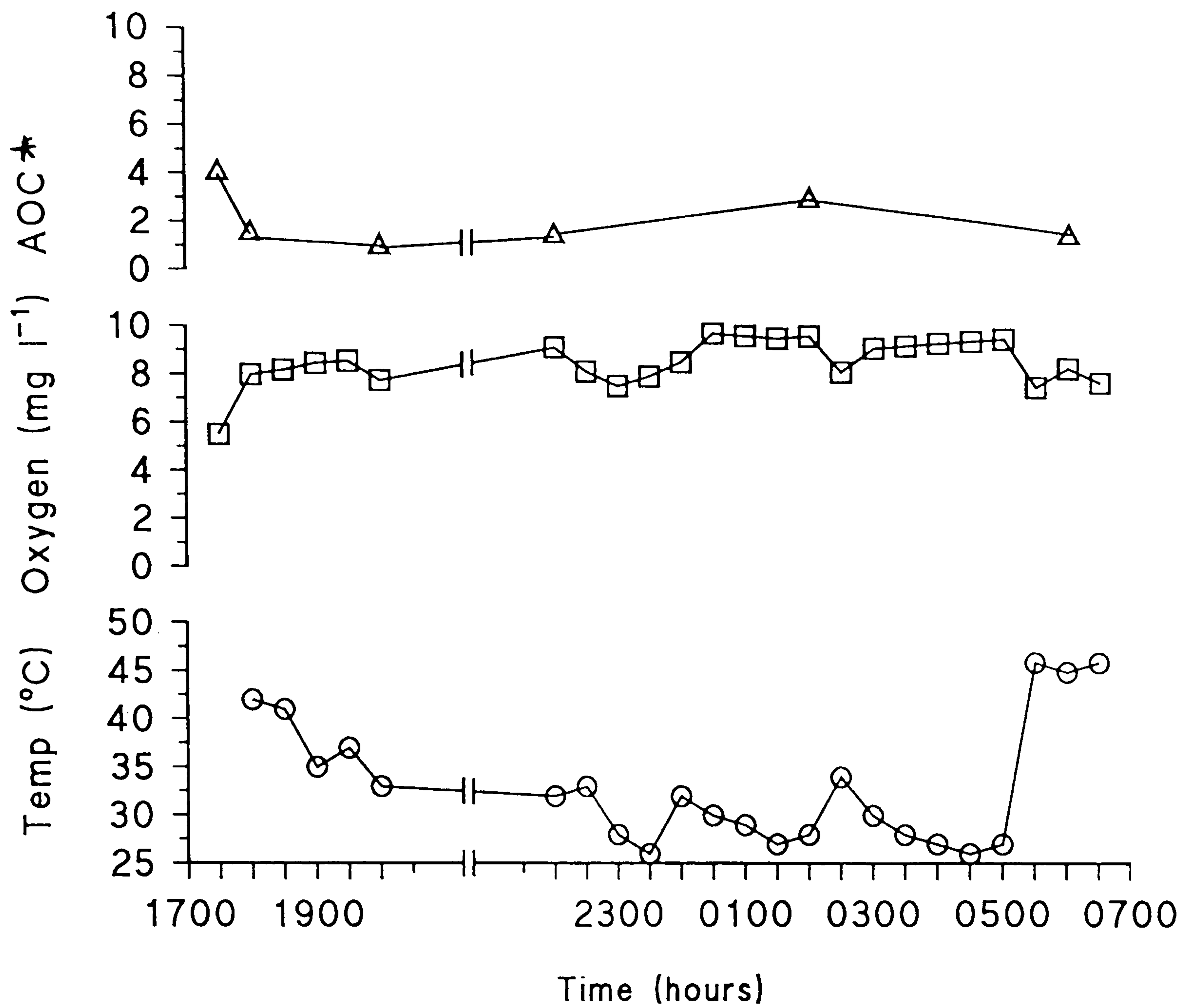


Figure 3.5 Continuous overnight monitoring of the temperature, dissolved oxygen and AOC at the Victoria Infirmary during the second survey in 1989. * denotes AOC as units of ATP.

(ii) Internal examination of the copper tubing.

When the water was extracted from the pipe it was found to contain a large quantity of dark brown flocculate deposits. This soft water was found to have a total hardness of $<20 \text{ mg l}^{-1}$ (as CaCO_3). Sulphides were present at $< 0.01 \text{ mg l}^{-1}$ and chloride at $< 10 \text{ mg l}^{-1}$. The internal surface was covered in a series of tubercles and green copper carbonate which was distributed over the entire surface (Fig. 3.6a). One particular tubercle can be seen in Fig 3.6b and when this tubercle was removed with 6M HCl the characteristic pepper pot-pitting was evident (Fig. 3.6c).

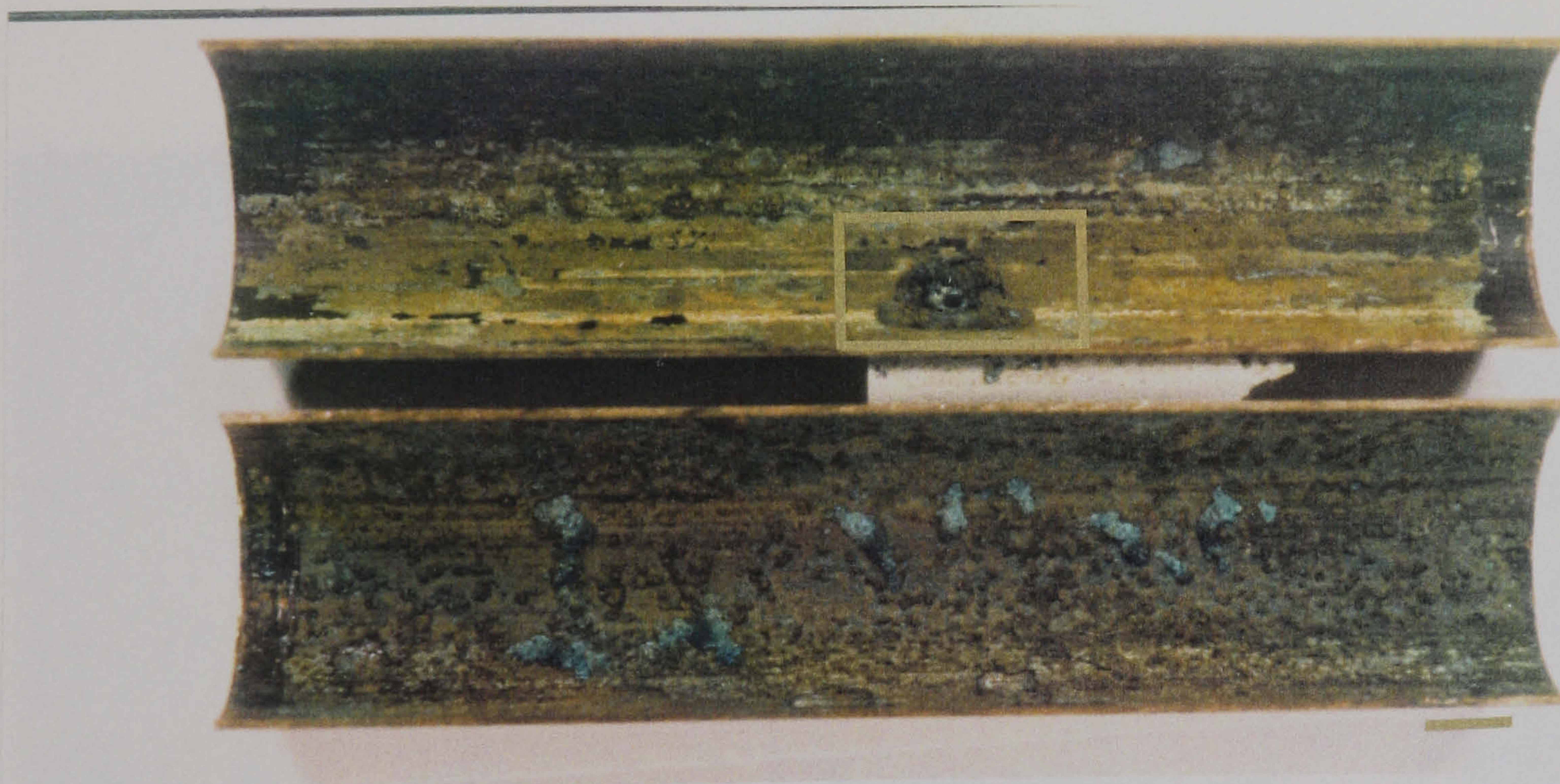


Figure 3.6a The internal surface of a section of copper tubing illustrating green copper carbonate corrosion products (Marker bar denotes 10mm).



Fig. 3.6b A large tubercle present on the copper tube surface as shown in the enclosed area in Fig 3.6a (Marker bar denotes 10 mm).



Fig. 3.6c Exposed view of etched copper surface illustrating pepper pot-pitting corrosion (Marker bar denotes 1 mm; C denotes the copper surface and P denotes the pits).

(iii) Microbiological analysis of the water and copper tube surfaces.

There was a total of 5.4×10^4 cfu cm⁻² from the water that was transported within the pipe section of which the *Pseudomonas* spp. and *Methylobacterium* spp. each represented 5 % with the other Gram-negative bacteria (OGN) being present at 90 % (Table 3.5 and 3.6, page 106/7). From the surface of the pipe section 2.8×10^3 cfu cm⁻² were recovered, however, *Pseudomonas* spp. represented 83 % and *Methylobacterium* spp. 17 % with no OGN being recovered.

3.3.2.2 Site C, Inverclyde Hospital.

Corrosion in this particular site had been reported by the on-site plumbers as being of the pepper pot-pitting type and the number of failures were recognised as significant. Although failures had been reported to be occurring predominantly in the hot water circuit, they had also been cited as the reason for failures in the cold water system. The incoming mains water was sampled from a drain valve in the basement (floor A) and was found to be at 9.4°C, pH 8.4 and DO 8.4 mg l⁻¹. Hot water was produced by direct fire heaters and recirculated round the hospital. No mixer taps were found in this building and the water was maintained at a temperature which would reduce the possibility of scalding of patients and staff. The temperature of the water from the first tap after the direct fire heater was 47°C.

(i) Continuous monitoring of the temperature, oxygen and AOC of the hot water supply.

The monitoring equipment was set up at a hot water faucet farthest from the direct fire heater in level L. Throughout the night the temperature of the hot water varied between 45°C and 50°C (Fig. 3.7). Monitoring commenced at 1700 h when the temperature of the water was 47°C. This then decreased to 45°C at 20.30 h before rising to 48°C at 22.30 h. The temperature was then maintained at 46-48°C until 07.00 h. Initially the oxygen concentration was 8 mg l⁻¹ at 17.00 h but by 17.30 this

had decreased to 5 mg l⁻¹ before stabilising at 6 mg l⁻¹. By 03.00 h it had increased to 7 mg l⁻¹ then steadily decreased to 4 mg l⁻¹ until 06.30 h when it increased to 8 mg l⁻¹ at 07.00 h. The AOC was initially 6.0 units of ATP at 07.30 and decreased to 1.0 unit of ATP 20.30 h. At 01.30 h it was 8.0 units ATP with 8.5 units of ATP detected at 07.00 h

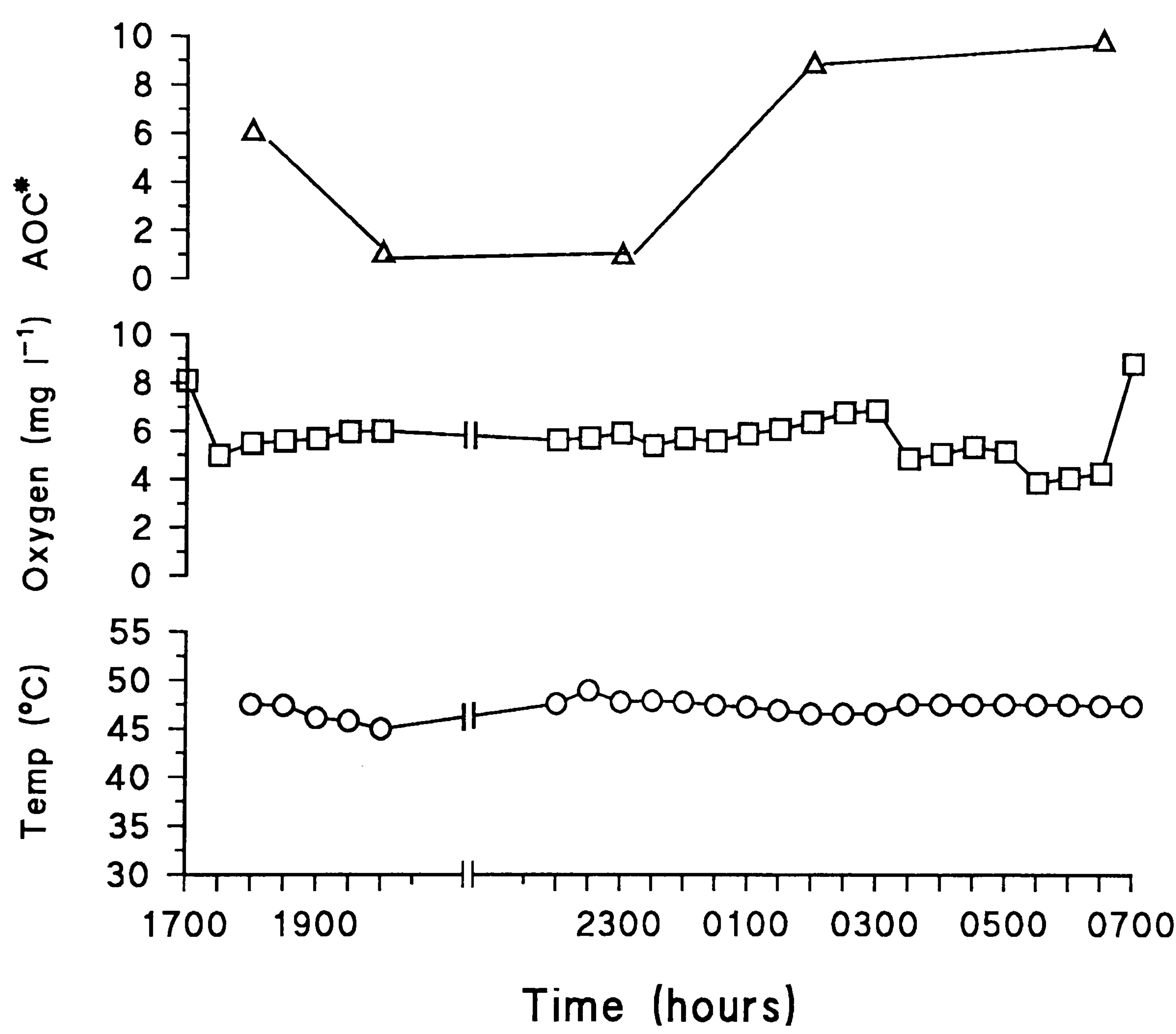


Figure 3.7 Continuous overnight monitoring of the temperature, dissolved oxygen and AOC at the Inverclyde hospital during the second survey in 1989. * denotes AOC as units of ATP.

(ii) Internal examination of the copper tubing.

The inner surface of the pipe which was extracted from a horizontal section of the plumbing of this hospital was completely covered in a thick brown coating which when scraped away exposed the copper carbonate corrosion products beneath (Fig. 3.8). Pitting was not evident on the inner surface of the pipe.

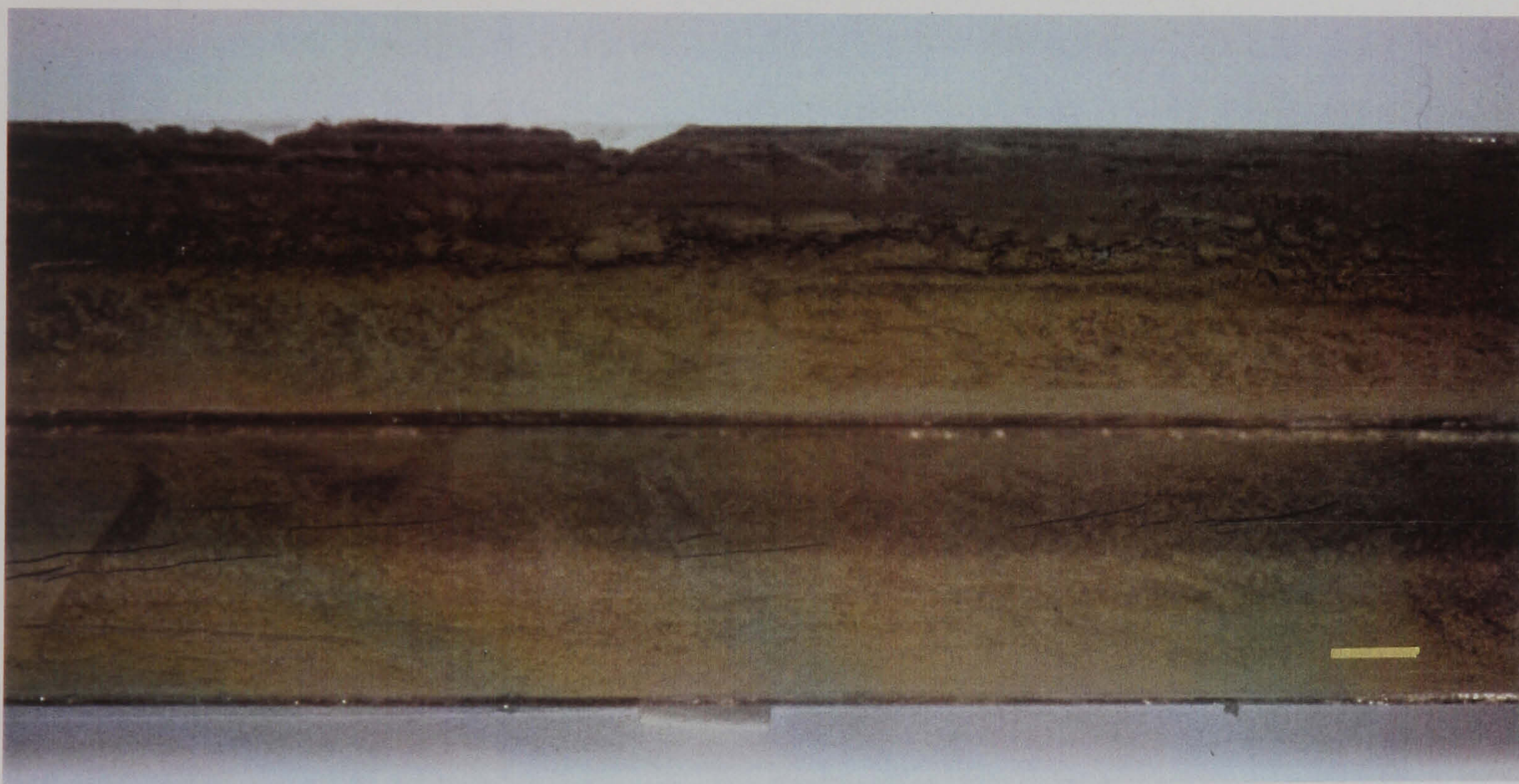


Figure. 3.8 Internal surface of the copper tube removed from the Inverclyde hospital during the second survey in 1989 (Marker bar denotes 10 mm).

(iii) Microbiological analysis of the water and copper tube surfaces.

The water taken from the inside of the pipe from site B contained a large amount of dark brown flocculate deposit. The number of bacteria recovered from the pipe surface and the planktonic phase was greater than 1.0×10^4 cfu cm⁻² and ml⁻¹ (confluent growth from maximum dilutions) respectively with other Gram-negative bacteria predominating (Table 3.5 and 3.6, pages 106 and 107). Sulphate-reducing bacteria were recovered from the internal surface of the pipe taken from this site.

3.3.2.3 Site D, Eastern General Hospital, Edinburgh, Scotland.

This was one of the control sites which had been chosen because pepper pot-pitting had not been reported. The steam-heated calorifier in this hospital was maintained at 55-60°C, with most of the water recirculated at this temperature. An energy management system monitored and controlled the water temperature at various locations in the hospital. All the faucets in the main hospital complex had mixer controls fitted which lowered the temperature of the water to 40-45°C to minimise scalding of patients and personnel. A non-recirculating section was chosen for monitoring as this was infrequently used and would represent the worst case of possible copper deterioration. The vertical copper pipe (approximately 10 years old) from which a 0.5 m section was removed was found to be neatly lagged and labelled.

(i) Continuous monitoring of the temperature, oxygen and AOC of the hot water supply.

The particular oxygen probe in the Sonde apparatus was found to be vulnerable to damage at temperatures greater than 50°C and so to prevent damaging the probe the water from the faucet in the kitchen department was adjusted to 45°C. The artificially reduced temperature was initially 43-45°C and decreased steadily throughout the night to 31°C the following morning (due to the thermostat being turned down to protect the DO probe) and may have had an influence on the other measurements (Fig. 3.9). The DO was initially measured at 12 mg l⁻¹ but immediately decreased to 8 mg l⁻¹ and was maintained at this concentration for the remainder of the monitoring period. Initially the AOC was recorded at 2.3 units of ATP before decreasing to 1.8 and 1.2 units of ATP at 19.00 h and 03.00 h before increasing again to 2.0 units of ATP at 07.00 h.

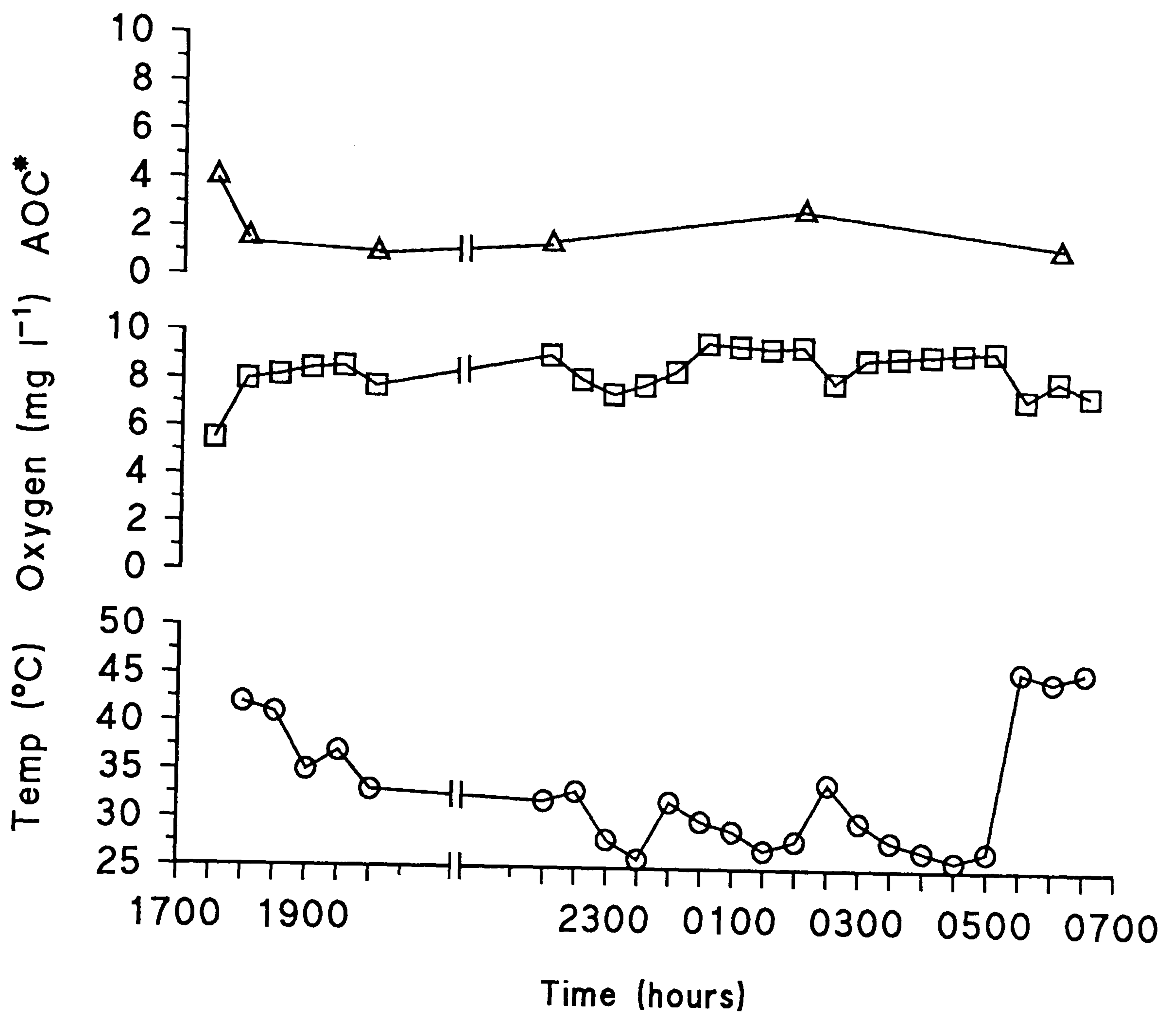


Figure 3.9 Continuous overnight monitoring of the temperature, dissolved oxygen and AOC at the Eastern General Hospital during the second survey in 1989. * denotes AOC as units of ATP.

(ii) Internal examination of the copper tubing

The vertical section of copper tubing which was removed from this hospital was found to contain a thin patchy layer of brown material on its surface with no tubercles or pitting attack taking place (Fig. 3.10).

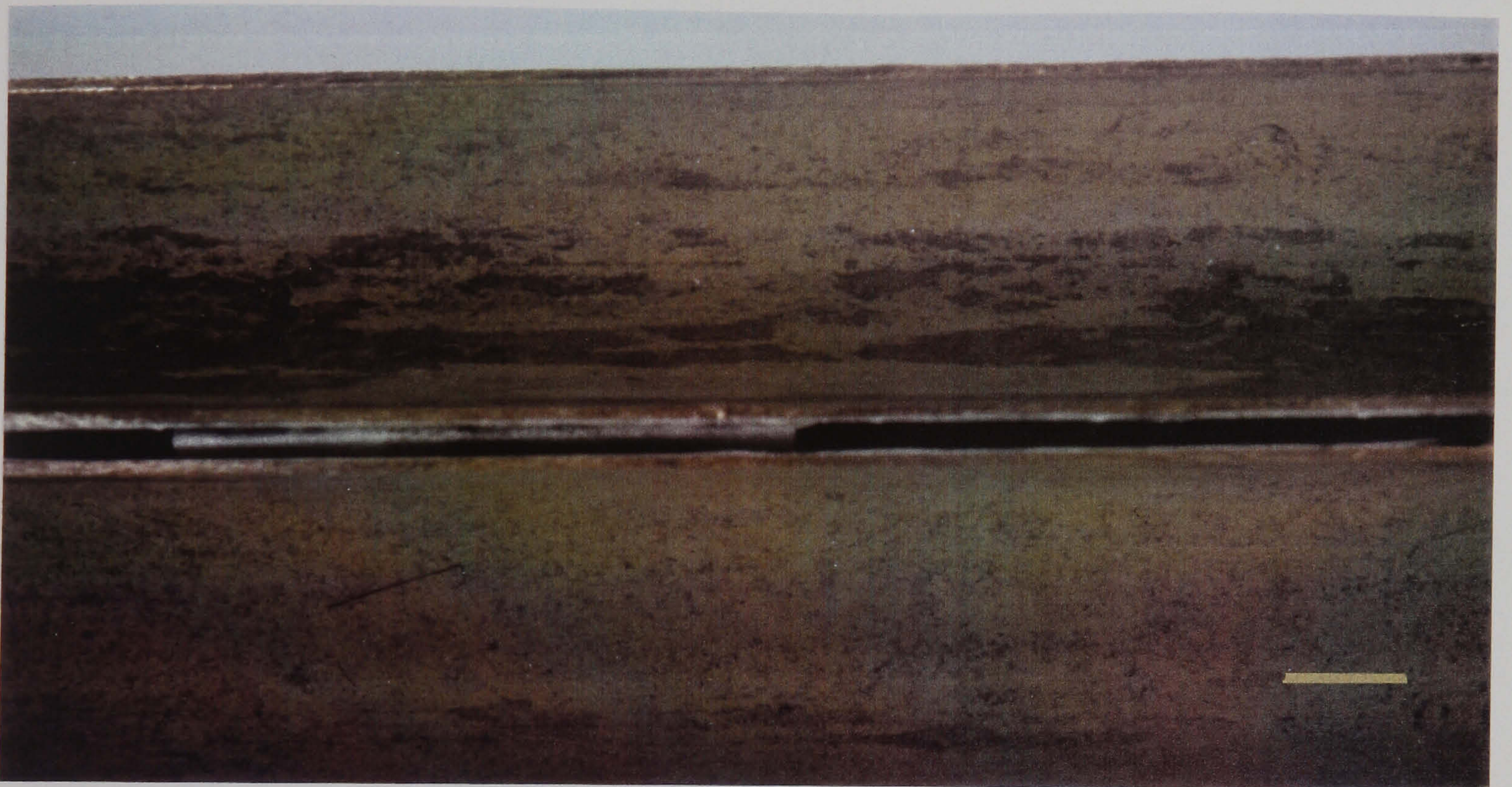


Figure 3.10 Internal surface of the copper tube removed from the Eastern General Hospital during the second survey in 1989 (Marker bar denotes 10mm).

(iii) Microbiological analysis of the water and copper tube surfaces.

A total of 1.4×10^4 cfu cm⁻² were recovered from the thin patchy brown material on the inner surface of the copper pipe with *Pseudomonas* spp. dominating the other Gram-negative bacteria found to be present (Table 3.5 and 3.6, page 106/7). As expected with little material being observed on the surface, there was only a small amount of brown deposit material present in the water. Only 1.6×10^4 cfu ml⁻¹ were recovered from the water phase with other Gram-negative bacteria predominating over *Pseudomonas* spp. However *Methylobacterium* spp. were found to be present in the water phase but not in the sample taken from the surface of the pipe. No SRB were recovered from this particular hospital.

3.3.2.4 Site E, Stratheden Hospital

This hospital was located near Cupar on the East coast of Scotland. The calorifier was heated to between 55-60°C and water was distributed on a recirculating system to all the units of the hospital. Mixer valves were fitted at the faucets to lower the temperature to 43°C, again to minimise scalding of patients and personnel. Pepper pot-pitting corrosion had not been reported at the Stratheden hospital.

(i) Internal examination of the copper tubing.

The copper tubing was removed from the hot and cold circuits of a relatively new children's block as it had already been reported as having failed in both the hot and cold circuits. When examined the hot and cold copper tubing were found to be running in parallel and close together with no evidence of lagging resulting. Upon examining the internal surface of the pipes both the hot and the cold (Fig. 3.11) were found to be covered in a thin patchy layer of material upon the surface between which the copper surface itself could be observed, with no evidence of the pepper pot-pitting corrosion.

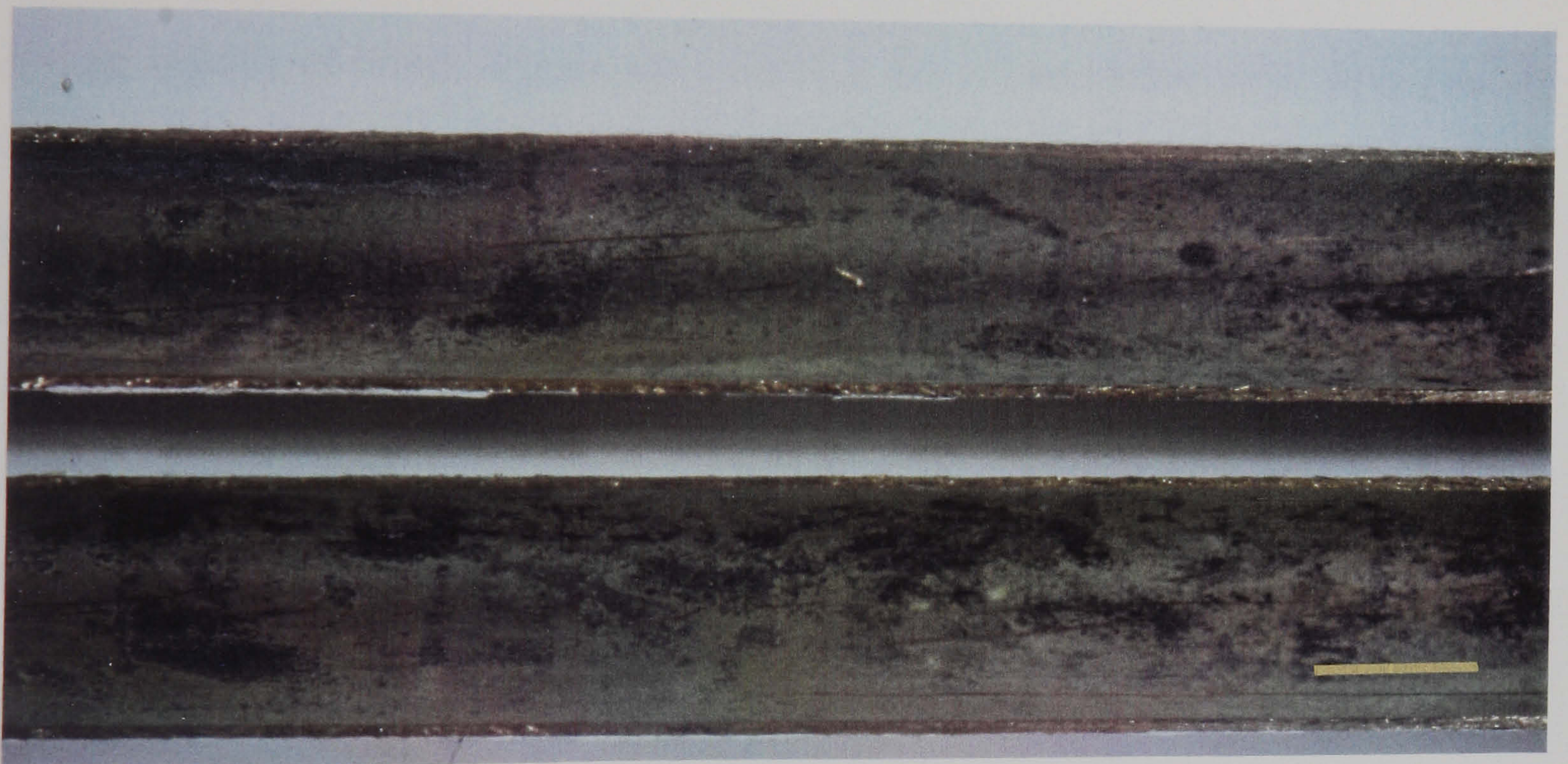


Figure 3.11 Internal surface of the copper tube removed from the Stratheden Hospital during the second survey in 1989 (Marker bar denotes 10 mm).

(ii) Microbiological analysis of the water and copper tube surfaces.

The water sample from the copper tube extracted from the hot water circuit was found to be relatively clear with only a slight brown tinge. Bacterial numbers recovered from this relatively colourless water sample were 1.6×10^4 cfu ml⁻¹ with only other Gram-negative organisms being identified. Only 2.8×10^3 cfu cm⁻² were recovered from the surface of the copper pipe. Of this *Pseudomonas* spp. represented 16% of the population with the majority being other Gram-negative bacteria.

A large quantity of brown deposit was present in the yellow-tainted water from the copper pipe removed from the cold water circuit. 1.6×10^4 cfu ml⁻¹ were obtained from the water phase of this pipe which was the same number of bacteria as from the water sample taken from the hot water circuit. *Pseudomonas* spp. represented 3% of this population with the remainder being other Gram negative bacteria. 2.8×10^3 cfu cm⁻² were recovered from the surface of the cold water pipe which was also the same as that in the hot water sample. However in the biofilm from this cold water pipe *Pseudomonas* spp. pre-dominated at 60 % of the population with the rest being other Gram negative bacteria.

(iii) Continuous monitoring of the temperature, oxygen and AOC of the hot water supply.

The hydrolab surveyor II was operated in a toilet cubicle of the nurses home and was supplied with hot water from a shower head. The temperature of the shower unit was adjusted to a constant 44°C throughout the monitoring period may have had an effect on the other measurements taken (Fig. 3.12). The DO measured a constant 8.0 mg l⁻¹ and the AOC 0.8 units of ATP.

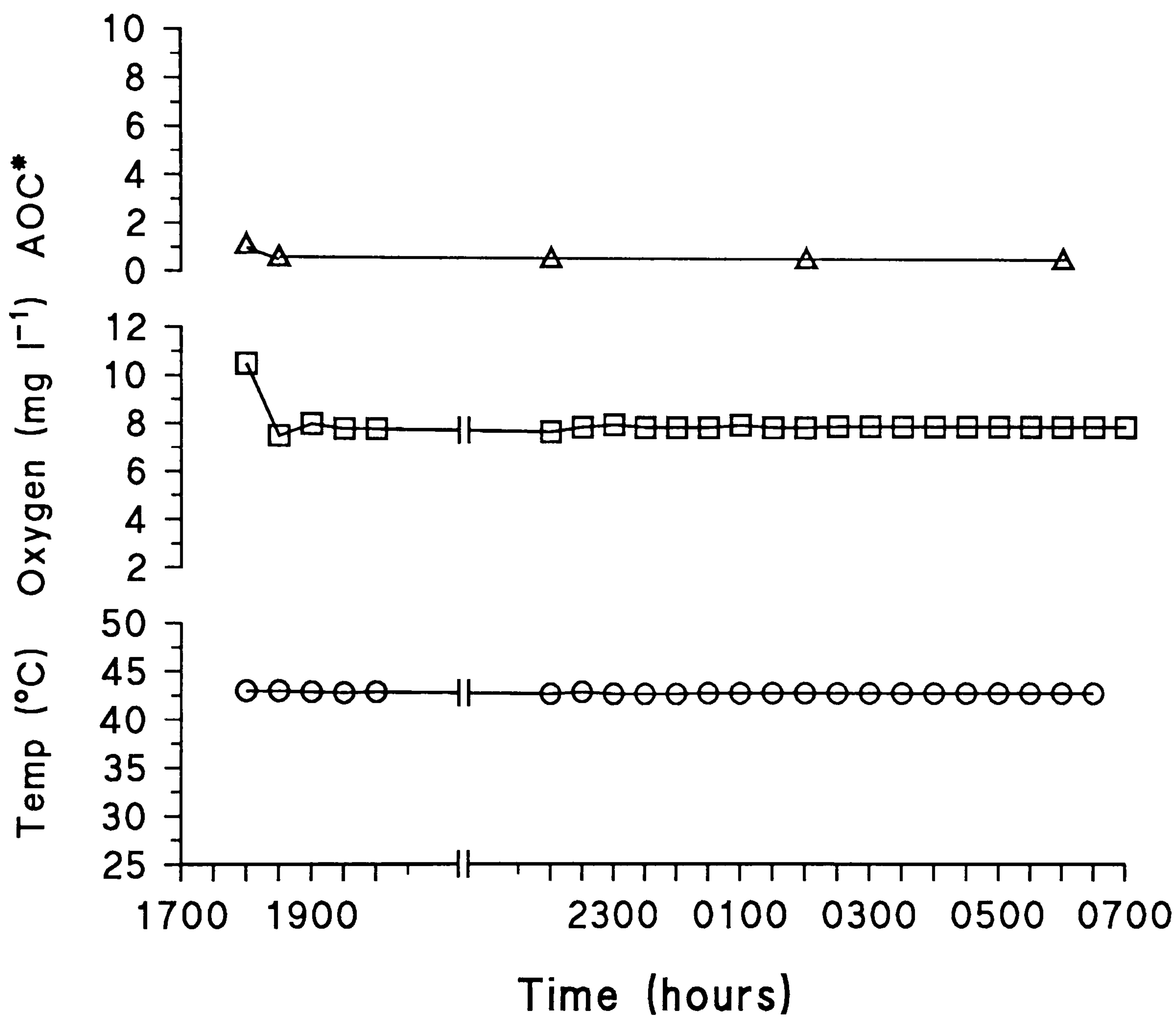


Figure. 3.12 Continual overnight monitoring of the dissolved oxygen and AOC of the hot water at the Stratheden Hospital during the second survey in 1989. * denotes AOC as units of ATP.

Table 3.5 Bacteriology and physical characteristics of copper tubes extracted from the hospital sites.

Site	Cal. Temp(°C)	Plank. Phase (cfu ml ⁻¹)	Biofilm Phase (cfu cm ⁻²)	Brown Deposit
Victoria (Hot)	48	5.4 x 10 ⁴	2.8 x 10 ³	+++++
Inverclyde (Hot)	47	>1 x 10 ⁴	>1 x 10 ⁴	+++++
Eastern Gen. (Hot)	60	1.6 x 10 ⁴	1.4 x 10 ³	+
Stratheden (Hot)	60	1.6 x 10 ⁴	2.8 x 10 ³	+
Stratheden (Cold)	9.9	1.6 x 10 ⁴	2.8 x 10 ³	++

Cal. denotes calorifier and Plank denotes planktonic. Bacterial determinations were obtained from a single sample.

Table 3.6 Representative ratios of bacteria from copper tube from the second survey.

Victoria(Hot)	GN ^a	<i>Ps</i> spp. ^a	<i>Meth</i> spp. ^a	SRB ^b	Leg. ^a
Biofilm	0	83	17	-	0
Planktonic	90	5	5	-	0

Inverclyde (Hot)	OGN ^a	<i>Ps</i> spp. ^a	<i>Meth</i> spp. ^a	SRB ^b	Leg. ^a
Biofilm	100	0	0	+++	0
Planktonic	100	0	0	+++	0

East. Gen. (Hot)	GN ^a	<i>Ps</i> spp. ^a	<i>Meth</i> spp. ^a	SRB ^b	Leg. ^a
Biofilm	33	67	0	-	0
Planktonic	50	40	10	-	0

Stratheden (Hot)	OGN ^a	<i>Ps</i> spp. ^a	<i>Meth</i> spp. ^a	SRB ^b	Leg. ^a
Biofilm	100	0	0	0	0
Planktonic	84	16	0	-	0

Stratheden (Cold)	GN ^a	<i>Ps</i> spp. ^a	<i>Meth</i> spp. ^a	SRB ^b	Leg. ^a
Biofilm	40	60	0	-	0
Planktonic	97	3	0	-	0

a, percentage; b, denotes presence: -, absence of SRB; + presence of SRB. nt denotes not tested. OGN denotes other Gram-negative bacteria; *Ps. spp.* denotes *Pseudomonas spp.*; *Meth. spp.* denotes *Methylobacterium spp.*; Leg denotes *Legionella spp.*

3.4 Discussion

A total of five hospitals were investigated during the two site surveys that were carried out in 1987 and 1989. The Victoria Infirmary and Glasgow Royal Maternity were reported to be suffering from pepper pot pitting corrosion of their copper pipework and were investigated in 1987. The Victoria Infirmary and Inverclyde Hospital, reported to be suffering from this unusual form of copper corrosion, were visited in 1989. Two other sites (Stratheden and Eastern General Hospitals) were also inspected in 1989 and were regarded as control sites as they had not reported a problem with pepper pot-pitting corrosion of copper tubing. However, corrosion of copper tubing was reported to be occurring in the hot and cold runs of copper tubing within a new children's ward of the Stratheden Hospital. When examined, it was found that the hot and cold tubes were running in parallel for about 5 metres with no evidence of lagging. Therefore, there was heat transfer between the pipes decreasing the temperature in the hot and increasing the temperature in the cold pipe which may have led to the observed corrosion which was not due to pepper pot-pitting.

3.4.1 Examination of incoming water and storage conditions

The incoming water was found to be 9.9°C at the hospitals reported to be suffering from corrosion of the copper tubing. In the water sample from the hydrant at the Victoria Infirmary only 200 cfu ml⁻¹ were detected with only 50 cfu ml⁻¹ at the Glasgow Royal Maternity. No coliforms were detected in any of the water samples tested indicating that the water was of an acceptable bacteriological standard for drinking water quality. Indeed only 1000 cfu ml were recovered from water samples taken from the tap furthest from the calorifier at the Glasgow Royal Maternity and in the nurses home at the Victoria infirmary. However a total bacterial number of 3.6 x

10^6 cfu ml⁻¹ including a *Legionella* spp., later identified as *Legionella bozemanii* by indirect immunofluorescent labelling, were detected in the base of the calorifier in the Glasgow Royal Maternity. This calorifier had been desludged and descaled approximately 12 months previously and *L. bozemanii* was only detected in the initial flush, which was measured at 27°C, from this calorifier indicating non-uniform heating. Further flushing resulted in water samples at 56°C with only 1000 cfu ml⁻¹ being recovered and no *Legionella* spp. in this hotter water. Thus, the reduced temperature in the calorifier permitted the growth of *L. bozemanii*, which has been associated with human pneumonia (Brenner *et al.* 1980), and a 3600-fold increase in the total number of bacteria, in comparison to water samples where the temperature was 56°C. The presence of *Legionella* spp within the base of the calorifier could indeed act as a seed for the rest of the water circuit where growth could resume in the water system where temperatures of 56°C could not be maintained.

Fungi were detected during the first survey in the cistern tanks and other samples taken from the calorifier at the Glasgow Royal Maternity but they were not detected in samples taken downstream of the calorifier. Fungal strains such as *Aspergillus* are known to produce gluconic and oxalic acids and have been shown to attack cement (Perfettini *et al.* 1989). Therefore, although fungi were not detected downstream of the calorifier their metabolic products which may also be aggressive to metal surfaces such as copper may well have been present. However, their presence in the cistern tanks and calorifier indicates the possibility of seeding the rest of the water circuit with fungi.

Other microorganisms which have been well documented in the corrosion of metals are sulphate-reducing bacteria (SRB) (Hamilton, 1985). SRB are strict anaerobes which grow by oxidising certain organic compounds or H₂ with sulphate or other sulphur compounds as terminal electron acceptors, and reduces them to sulphide ions which are highly reactive and corrosive to metals. SRB were found extensively at all but one

site at the Victoria Infirmary (tank sediment) examined in the first survey 1987 but no SRB were found at the Glasgow Royal Maternity. In the second survey in 1989 SRB were also detected in the pipe sample extracted from the Inverclyde Hospital but not at the Victoria Infirmary. Neither were SRB detected at the control sites, the Eastern General Hospital and Stratheden Hospital. Thus far, SRB have been detected in two out of the three hospitals which were reporting an unusual number of copper tube failures due to pepper pot-pitting. Low numbers of SRB have been implicated in the corrosion of copper/nickel piping systems even when sulphides could not be detected (Little *et al.* 1989), indicating that when present in low numbers they may have a role to play in corrosion .

As mentioned above the quality of the water supplying all the sites was found to be bacteriologically satisfactory for drinking purposes. However, the storage tanks at the Victoria Infirmary were found to be lacking a cover, allowing debris and peeling paint from the roof to fall into this tank which was supplying water to the calorifier. Indeed, this extraneous material falling into the open tank may have eventually added to the increase in the AOC from 7.4 units of ATP in the incoming water to 12.4 units of ATP (41 % increase) and the presence of 5.0×10^7 cfu per swab from the wall of the cistern tank. LeChevallier *et al.* (1987) also utilised AOC (according to the method of Van der Kooij *et al.* 1982) to indicate a 37 % increase in nutrient consumption between three sites in a distribution system. Even two years later these tanks were found to be in an unsatisfactory condition with badly fitting lids giving access to dirt and insects that would increase the organic loading of the water. At the Glasgow Royal Maternity the tanks were found to be sealed and in a very good condition. However, this was later found to be due to the tanks having been cleaned, painted in bitumen and chlorinated two weeks before the survey was carried out. Due to the short turnaround in restoring and reinstating the tanks, care has to be taken as bitumen paint requires a recommended curing period. If this curing was not carried out the paint may not operate as efficiently as a corrosion protective layer (Colbourne, 1985) and may even

leach nutrients into the water supply. The plumbers informed us that prior to refurbishment the tanks possessed a layer of slime on the inner walls with silt present on the bottom indicating problems with maintenance. Even after being refurbished 1.0×10^8 cfu per swab were recovered from the wall of this cistern tank, indicating that it was quickly being recolonised by the bacteria present, even though the tank looked to be in a very good condition. Refurbishment of the cistern tank may have accounted for the absence of SRB in the tank and after the hydrant, however, it would not account for their absence from the incoming water before the cistern tank.

Water was not stored at the Inverclyde Hospital, as the hot water was supplied by direct fire heaters. Upon visiting the two control sites, the Eastern General and Stratheden Hospitals, the water storage tanks were found to be clean and tidy and have the appearance of being maintained regularly.

3.4.2 Examination of the water system parameters

The Hydrolab Surveyor was utilised to monitor the temperature and dissolved oxygen of the hot water supply at the Victoria Infirmary (first and second survey), Inverclyde, Eastern General and Stratheden Hospitals. In the first survey at the Victoria Infirmary the temperature of the hot water was initially measured at approximately 50°C and started to decrease gradually from 17.30 h to approximately 40°C. The temperature recovered the following morning at 06.00 h. The dissolved oxygen concentration was also monitored overnight and followed a similar profile to the temperature. Initially the dissolved oxygen concentration was measured at approximately 8 mg l⁻¹ and shortly after 18.00 h it was found to decrease to virtually 0 mg l⁻¹ and only increased again at 06.00 h concurrent with the increase in the temperature.

In the second survey at the Victoria Infirmary the temperature was initially measured at

43°C at 17.30 h and then decreased to 27°C overnight before increasing again to 45-47°C at 05.30 h the following morning. Overnight the dissolved oxygen fluctuated between 7.0 and 9.0 mg l⁻¹ and there was no decrease as was observed during the first monitoring period at this site two years earlier. Refurbishment of the cistern tank may have accounted for the absence of SRB which are strict anaerobes and which were found at all but one site tested at the Victoria Infirmary two years earlier. Increasing the speed at which the hot water was recirculated to increase aeration and stabilise the water temperature was a recommendation from the first survey and would account for the constant aeration during monitoring in 1989. Increasing the aeration of the water by increasing the speed of the recirculation pumps could have resulted in a positive reduction potential which may discourage the growth of anaerobic SRB. Water samples were taken from the hot water supply periodically throughout the monitoring for analysis of the AOC. The AOC was initially measured at 4.0 units of ATP and decreased to 1.0 unit of ATP before increasing at 02.30 h to 3.0 and was measured at 1.5 unit of ATP at 06.00 h.

Monitoring of the water parameters at the Inverclyde Hospital revealed that the temperature was relatively constant at 45-47°C. However, the oxygen concentration, which was initially measured at 8.0 mg l⁻¹, decreased to between 5-6.0 mg l⁻¹ overnight before increasing to 9.0 mg l⁻¹ at 07.00 h. The AOC was measured at 6.0 units of ATP and decreased to 1.0 unit of ATP till 10.00 after which it increased to 9.0 units of ATP.

The temperature of the hot water from the calorifier at the Eastern General Hospital was between 55-60°C. Unfortunately one of the drawbacks of using a housing containing various probes to measure parameters in a hot water system is that the oxygen probes are not designed to operate at temperatures above 50°C. Where necessary the temperature of the hot water was decreased to prevent damage to the dissolved oxygen probe at the site of monitoring and so may have affected the other

measurements. This also occurred at the Stratheden Hospital and so the temperatures represented at both these sites are artefacts. Mixer valves were fitted to decrease the temperature to 47°C for the end user to minimise patient scalding. After initially being measured at 12.0 mg l⁻¹ the dissolved oxygen concentration at the Eastern General Hospital was constant at 8.0 mg l⁻¹ which was similar to the trend observed at the Stratheden Hospital. After being measured at 2.5 units of ATP the AOC at the Eastern General Hospital dropped to 1.5 units of ATP and then decreased to 1.0 unit of ATP before returning to 2.0 units of ATP at 17.00 h the following morning. At the Stratheden Hospital the AOC was measured at 1.0 unit of ATP at the start of the monitoring period and decreased to 0.5 units of ATP and did not increase again.

Maximum AOC concentrations were found at the Inverclyde Hospital and Victoria Infirmary at 9.0 and 4.0 units of ATP respectively, thus providing microorganisms within these water systems with a greater concentration of nutrients for metabolism and growth than in the Eastern General and Stratheden Hospitals, where only 2.0 units of ATP or less were detected.

3.4.3 Examination of the copper tubing surfaces

The copper tubing removed during the site surveys were brought back to the laboratory for examination. Copper tube removed from the Victoria Infirmary (both surveys) and the Inverclyde Hospital were examined by light and scanning electron microscopy as well as SEM and were found to have a thick glutinous layer associated with the surfaces. Using light microscopy and Gram-staining the glutinous layer was found to be composed of bacteria, predominantly Gram-negative rods. Tubercles were found to be present on the surface surrounded by a glutinous layer, and when the tubercles were scraped away a series of steep sided pits, typical of pepper pot-pitting were revealed. Previous examination of tubercles in distribution systems has revealed

that they were found to contain sulphate-reducing bacteria as well as a range of aerobic heterotrophic bacteria (Tuovinen *et al.* 1980) and that the tubercles exhibited a clear correlation with pitting corrosion in cast iron pipes. LeChevallier *et al.* (1987) also detected tubercles in water distribution pipelines and correlated them with a large number of bacteria, in particular, coliforms. Although Tuovinen and Hsu (1982) did not detect coliforms they demonstrated that tubercles present a niche in which bacteria can survive and even be protected against chlorination. However, when the copper pipes removed from the Glasgow Royal Maternity, Eastern General Hospital and the Stratheden Hospital were examined, they were found to have a thin layer of material on the surface with no indication of tubercles. From the microbiological quantification there was a higher number of microorganisms recovered from Inverclyde Hospital than from the other three sites. Although the same number of bacteria were recovered from the surface of the other three sites there was a greater number present in the water of the pipe from the Victoria Infirmary suggesting that bacteria may have sloughed off the surface in transit.

3.5 CONCLUSIONS

In summary, the surveys set out to identify reasons why particular hospitals were experiencing severe copper tube corrosion due to pepper pot-pitting and others were not. At the Glasgow Royal Maternity, Stratheden Hospital and Eastern General Hospital pepper pot-pitting corrosion of copper tube was not considered a problem, the temperature of the recirculating water circuit was 55°C with the latter two having a low AOC concentration. This relatively high temperature and low AOC concentration may have been the controlling factor in reducing the presence of bacteria within the water circuit, indicated by the thin layer of deposit and biofilm present on the surface of pipes removed from those sites.

The hospitals where pepper pot-pitting corrosion was present were the Victoria Infirmary and Inverclyde Hospital and the hot water circuit was monitored between 40-46°C. In the first survey in 1987 the AOC concentration at the Victoria Infirmary was 12 units of ATP and 4.0 units of ATP in the second survey in 1989 and 9.0 units of ATP at the Inverclyde Hospital. The presence of a high concentration of nutrients in the form of AOC and a relatively low temperature of between 40-46°C would allow the prolific growth of the heterotrophic bacteria present in the water circuit and on the copper pipe leading to a decrease in the DO as was observed during monitoring. The presence of tubercles and a thicker biofilm on the copper pipe surface of the latter two sites correlates with increased bacterial growth due to favourable conditions.

Parameters such as a relatively low temperature and high AOC concentration resulting in a decrease in the dissolved oxygen concentration and the presence of a thick biofilm correlates with the occurrence of pepper pot-pitting corrosion in the copper tubing. The aerobic facultative bacteria present in the biofilm on the copper surface would interact with the fungi present resulting in a complex consortia forming a framework in which the biofilm can grow. SRB where present in this complex biofilm network would establish within an anaerobic niche to enrich the complexity and aggressive nature of the biofilm on the copper surface.

The results were used to identify parameters which may have contributed to the encouragement of the pepper pot-pitting corrosion in order to establish and where necessary to modify the laboratory model. The primary aim of the model was to simulate the environmental parameters at the Victoria Infirmary to reproduce the fouling of the copper tubing and to develop methods to control or prevent such fouling. Temperature was chosen as the main parameter with which to focus on by establishing a laboratory model to simulate the conditions under which this particular corrosion was occurring.

In conclusion the hospitals which were examined had locations that were reporting failures in their copper tubing due to pepper pot-pitting corrosion. The water supplying these sites contained a relatively high concentration of assimilable organic carbon (AOC). As this water was from an upper catchment area (peatland) the water would have contained the breakdown products of humic and fulvic acids resulting in the increased AOC providing the nutrients for microbial growth.

Therefore, with the decrease in temperature of the water system overnight microorganisms present at the surface as a biofilm would become metabolically active with the resultant decrease in AOC and dissolved oxygen concentration due to bacterial metabolism and respiration.

Particulate matter in the water would become enmeshed in the biofilm resulting in microcolonies forming into tubercles. It is directly beneath these tubercles that the pepper pot-pitting corrosion was perforating the copper tubing.

The presence of the bacterial microcolony would result in the formation of electrochemical cells due to concentration gradients across the copper surface. In association with the physical structure of the microcolony resulting in concentration gradients the metabolic activity of the microorganisms would lead to an aggressive environment directly at the copper surface. In conclusion the metabolically active biofilm appears to have a direct role in the formation of pepper pot-pitting.

CHAPTER 4.0

LABORATORY MODELLING OF BIOFOULING.

4.1 INTRODUCTION

Bacterial association with copper tube had been observed in all the pipe samples recovered from the institutional buildings where corrosion was occurring (Chapter 3.0). In the sites where corrosion was reported to be a problem, operation of the hot water system at relatively low temperatures (46°C) would have stimulated a metabolically active bacterial biofilm. Such biofilm could have been responsible for the decrease in the AOC and the observed drop in the dissolved oxygen concentration. The aim of the laboratory model was to simulate the fouling of copper tube under conditions as close as possible to those found in hot water circuits where copper failures were occurring. For this purpose, water from the Victoria Infirmary was used as the medium in which to grow a bacterial inoculum, taken from the inside surface of a corroded copper tube obtained from the same site.

Many laboratory models have been developed to study bacterial growth (Chapter 1), but the important criterion is that one understands the limitations of the laboratory apparatus. A model cannot replace the real environment but can provide a controlled situation in which to study growth or a particular parameter which one deems as being important. In the present study a continually flowing water circuit and the ability of organisms to colonise the plumbing tube material (copper) under particular conditions had to be simulated.

To simulate the continually flowing water circuit it was proposed to set up two continuous culture laboratory vessels in series with the first vessel being utilised primarily to grow up an inoculum from the copper pipe surface. The stabilised culture in the first vessel would be grown and used to continuously feed a second vessel, where copper coupons would be immersed in the culture, thus simulating attachment to copper pipes in a continually flowing water circuit. With coupons suspended in the

second vessel, parameters could be altered, even to the detriment of the culture in that particular vessel to determine the effect on it of variable parameters. If the culture was diminished then when conditions became favourable growth would result as the vessel would be continually challenged with culture from the first vessel where conditions are consistent. Initially the apparatus was utilised to simulate the fouling of copper tube at different temperatures to determine whether the profile of biofouling in the hot water system could be reproduced in the laboratory. Following this, the effect of temperatures greater than 60°C was investigated on developing and developed biofilms, as well as bacteria in the planktonic phase.

Copper tubing in domestic and institutional buildings is guaranteed for 25 years however a small number of failures have been reported in a few isolated cases within this guarantee period. Water supplied to domestic and institutional buildings is not sterile (Gibbs and Hayes, 1988) and contains a range of microorganisms such as *Pseudomonas* spp., *Methylobacterium* spp. and *Flavobacterium* spp. as well as opportunistic pathogens such as *Aeromonas* spp. The detrimental effect of biofilms has been documented particularly in reference to SRB and MIC of mild steel (Hamilton, 1985). The type of copper tube failure identified in the present study was unusual and bacterial involvement had been suspected following a number of independent studies (Fischer *et al.* 1988; Chamberlain *et al.* 1988; Keevil *et al.* 1987) and site surveys (Walker *et al.*, 1991) (see Chapter 3). Control measures to eradicate biofilm using acid treatment has been investigated (Fischer *et al.* 1992). Reiber *et al.* (1987) successfully demonstrated a 40 % reduction in the corrosion of copper plumbing material by increasing the hardness of the water to passivate the copper tube surface. Therefore a laboratory rig was also established involving two continuous culture vessels separated by a 10 m length of copper tubing. Soft water containing particulate matter was supplied to the first vessel and copper tube but the water was then passed through an inline cartridge filter before it was pumped into the second vessel, thus allowing for a comparison of particulate matter on biofilm development.

Visualisation of bacterial attachment and growth has been extensively studied by Caldwell and Lawrence, (1986) using light microscopy. In this present study a light microscope with adapted differential interference contrast (DIC) and UV-fluorescence was also utilised to view bacteria on glass surfaces that had been immersed in the laboratory model as a control to gauge the amount of biofouling against other materials. The glass surface being flat presents an ideal surface on which to view, using DIC, the biofouling taking place. However, materials used in domestic plumbing circuits and cooling towers are generally opaque with only a few of the plastic materials allowing transmissible light to pass through them, albeit with some difficulty. Even so, the plastics present a relatively smooth flat inner surface to view biofouling by DIC. The larger the diameter of the pipe, the less the curvature and the greater the amount of area which will be in view in the focal plane. It is in circumstances where curved pipe has to be examined for evidence of biofouling that the long focal length non-contact metallurgical lenses are extremely useful. DIC is superior to phase contrast microscopy and, since they do not need any prior preparation, samples can be observed rapidly without the introduction of artefacts. DIC is commonly used with transmitted light but biofilms may be dense and occur readily on opaque materials.

For material such as copper and other metals the biofilm on the surface can be examined with DIC but the individual bacteria are often difficult to visualise. For this reason fluorochromes such as acridine orange have been utilised to observe the individual bacteria and microcolonies on copper and to enhance visualisation on glass. For counting purposes the use of microscopical techniques to obtain direct total and viable counts in a short period of time can be very informative. Acridine orange (Daley and Hobbie, 1975) has been used to enumerate total bacterial numbers but the resulting range of colours from yellow to green of the individual bacteria does not necessarily equate to viability especially on heat treated cells at 60°C (Back and Kroll, 1991). Hobbie *et al.* (1977) suggest that the most active and dead bacteria will

fluoresce red and inactive or slow growing bacteria will fluoresce green. However, it has been used successfully to identify intracellular from extracellular enteropathogens in Hela cells (Miliotis, 1991). One of the disadvantages of the technique is the quenching or fading of the fluorescence.

An established technique of investigating viable bacteria is to use tetrazolium salts such as 2-(*p*-iodophenyl)-3-(nitrophenyl)-5-phenyl tetrazolium chloride (INT) (Zimmerman *et al.* 1978). Respiring bacteria that possess an active electron transport system will reduce INT to INT-formazan. Thus INT can be used in conjunction with other stains to distinguish viable bacteria from the total number of bacteria viewed microscopically. Vesey *et al.* (1990) combined an immunofluorescent assay (IFA) with INT for the rapid identification and enumeration of viable *Legionella pneumophila* serogroup 1. In that study IFA was used to obtain the total number of *L. pneumophila* serogroup 1 while INT was used to indicate viable cells.

The present study incorporated the use of a light microscope (Nikon Labophot 2) equipped with episcopic DIC to view opaque materials. A mercury lamp combined with UV-fluorescence and immuno-gold blocks facilitated immunolabelling studies. Key features of this particular microscope were the long focal length, non-contact metallurgical objectives that avoided the need for coverslips or oil contact with specimens. Therefore reflected episcopic light or fluorescence was used to view biofilms using procedures that attempted to keep biofilm distortion to a minimum.

Two major techniques were chosen to study corrosion and biofilm development on samples extracted from the laboratory rig. These were environmental SEM (ESEM) and confocal laser microscopy.

ESEM allows the structure of the biofilms to be examined without prior preparation of the sample, thus preventing the introduction of artefacts such as shrinkage due to

fixation, or loss of sample due to frequent changes of buffers and dehydrating agents (Chang and Rittman, 1986). The procedure operates on the principle of an SEM, however the specimen chamber is maintained under higher pressures so that hydrated samples can be viewed in their natural wet environment. As the specimen is maintained at approximately 6 torr pressure then altering the pressure in the chamber will alter the amount of water which surrounds the specimen e.g. decreasing the pressure will increase the sublimation of water from the surface. Although of primary use in the food industry it is at present gaining favour among biologists (Danilatos, 1991; Little, 1991). Confocal laser microscopy has enabled the structure of multi-species biofilms to be examined (Cummins *et al.* 1992) and in the present study this apparatus was utilised to obtain a better understanding of biofilm and its affect on the metallic substrata. Thus from developing the laboratory model to simulate a particular problem in a water system, techniques were developed to study colonisation, biofilm development and control as well as to investigate possible corrosion mechanisms that may be occurring.

4.2 MATERIALS AND METHODS

The laboratory model was based upon two continuous culture vessel linked in-series to simulate a flowing water circuit. Using this type of model the first vessel was used to simulate a water storage tank feeding the water circuit down-stream, that is, the second vessel. Materials, such as glass, silicone rubber and titanium, used in the design of the model (section 2.5, page 51) were chosen due to their inert nature. These materials would not leach or impart metals or nutrients into the culture. Release of such compounds may otherwise influence the chemistry of the water and/or bacterial growth in the vessel. An environmental inoculum source of micro-organisms was obtained from the surface of a corroded copper pipe (section 2.4, page

50) and inoculated into the first vessel. Biofilm development was ascertained by immersing plumbing tube materials (coupons) into the second vessel and removing coupons from the vessel at selected time periods (section 2.7, page 58). Conditions within the second vessel were altered to determine the effect of temperature and water chemistry (carbonate concentration) (sections 2.7.1 and 2.7.4, page 59) on biofilm development. To determine the effect of cleaning agents to remove biofilms, coupons were taken from the culture vessel and treated with either citric (10 %) or sulphamic (5 %) acid (section 2.7.3, page 60). The coupons were then re-immersed into the culture vessel to assess recolonisation. To complement standard plate count procedures to evaluate viable cell numbers, various microscopy techniques (light microscopy, DIC, epi-fluorescence, SEM and ESEM) were used to visualise the morphology of the biofilms (section 2.12, page 64). Further microscopy studies, using a scanning confocal laser microscope, were carried out to evaluate the effect of particulate material and bacterial metabolism on the corrosion of copper coupons (section 2.12).

4.3 RESULTS

4.3.1 Simulation of environmental conditions causing corrosion

Background

The previously described results obtained during the site surveys indicated that the temperature of the hot water circuit in the corroded site fluctuated between 34-52°C overnight (Fig. 3.0, page 78). The continuous culture laboratory model was set up to simulate this temperature cycle and investigate fouling of copper coupons over 40-60°C. The culture inoculum was established at 40°C in the filter sterilised tap water and maintained at 40°C with specimen material coupons immersed in the vessel for 21 days. Biofilm generation over the temperature range of 40-60°C was examined by increasing the temperature by 5°C increments 7 days after each 21 day biofouling experiment.

4.3.2 Biofilm development of copper surfaces

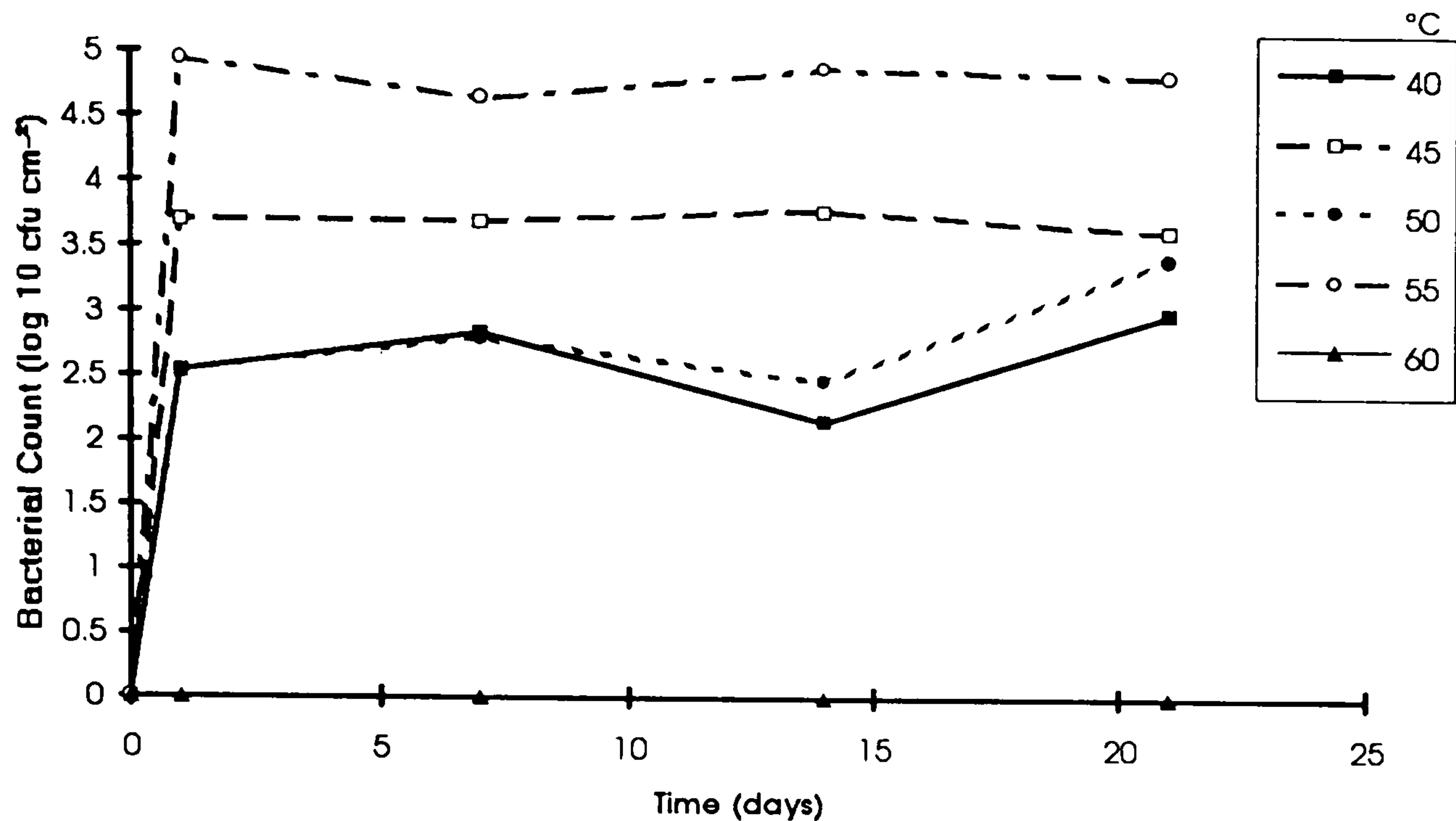


Figure 4.0 Colonisation of copper in Glasgow water between 40-60°C.

Colonisation of the copper surfaces occurred rapidly within 24 h of the copper coupons being placed within the culture over the temperature range of 40-55°C. During this period recovery of viable bacteria ranged between 2.5 (40°C and 45°C) and 4.9 (55°C) Log 10 cfu cm⁻². Although similar numbers were obtained from coupons at 40°C & 50°C (2.5 Log 10 cfu cm⁻²), greater numbers of viable bacteria were recovered at 45°C (3.7 Log 10 cfu cm⁻²) and 55°C (4.9 Log 10 cfu cm⁻²). As time proceeded the number of bacteria recovered from 45 and 55°C remained stable while those recovered from 40 and 50°C increased from 2.5 Log 10 cfu cm⁻² to 2.9 and 3.4 Log 10 cfu cm⁻² respectively. In comparison when the temperature was increased to 60°C no viable bacteria were recovered from the coupons. There was no significant difference between the number of bacteria obtained at 40 & 50°C ($p = >0.05$). In contrast the differences between 40 & 45°C, 40 & 55°C, 45 & 50°C, 45 & 55 °C and 50 & 55 °C ($p = <0.05$) were significant.

4.3.2.1 Profiles of the microbial types recovered from copper (40-60° C)

(i) Percentage profile of microorganisms on copper at 40°C.

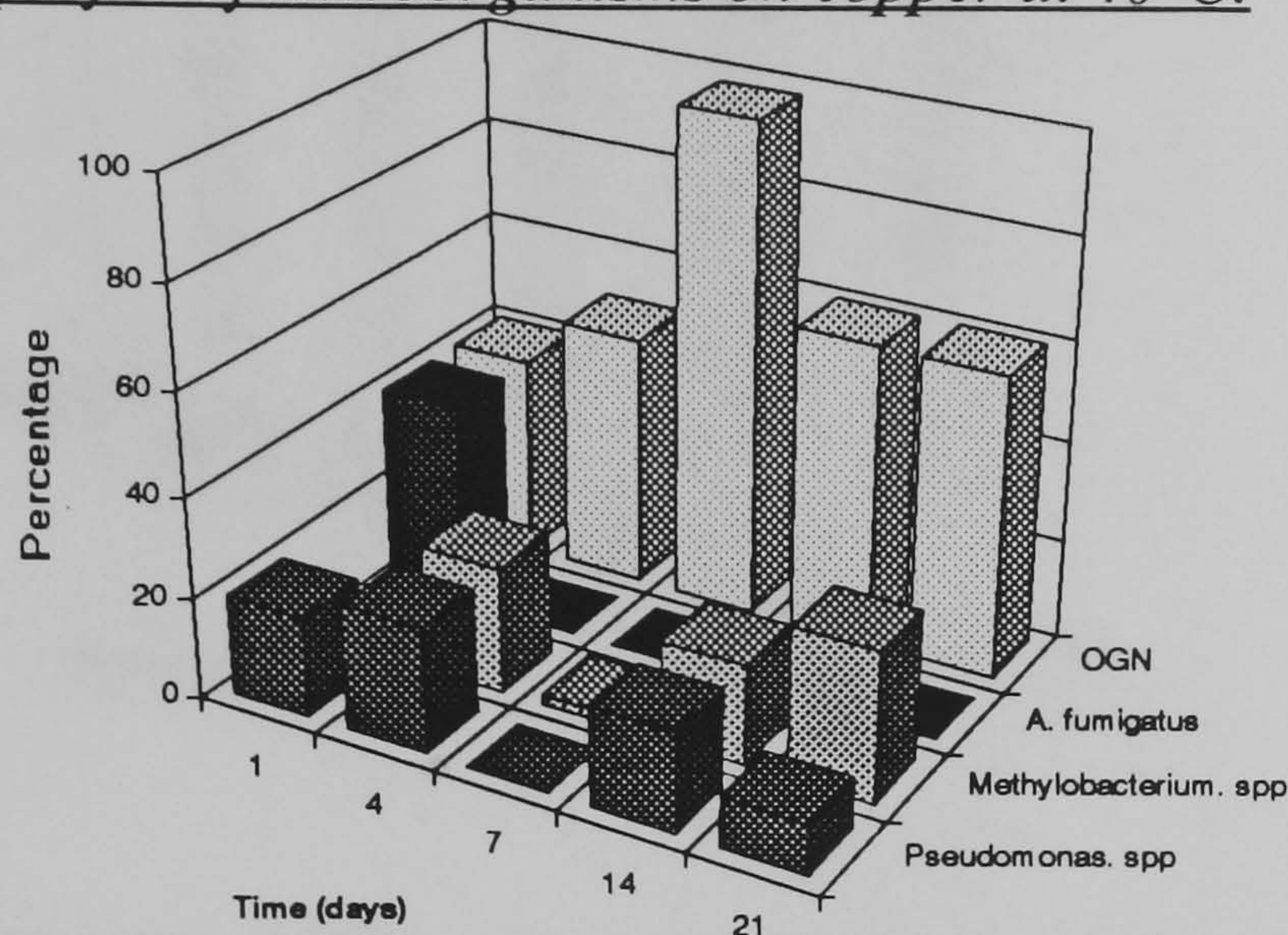


Figure 4.1 Percentage profile of microorganisms on copper at 40°C.

Table 4.0 Percentage profile of microbial species on copper at 40°C.

Species / Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	20	25	0	20	10
<i>Methylobacterium</i> spp.	0	25	2	20	30
<i>Aspergillus fumigatus</i>	40	0	0	0	0
Gram Negative Bacteria	40	50	98	60	60

Aspergillus fumigatus was only detected at day 1 at 40 %. *Pseudomonas* spp. were recovered on all but day 7 between 10-25 %. *Methylobacterium* spp. were not recovered on day 1 but were detected at between 2-30 % thereafter, where as the OGN dominated throughout the time course at 40% or greater.

(ii) Percentage profile of microorganisms on copper at 45°C.

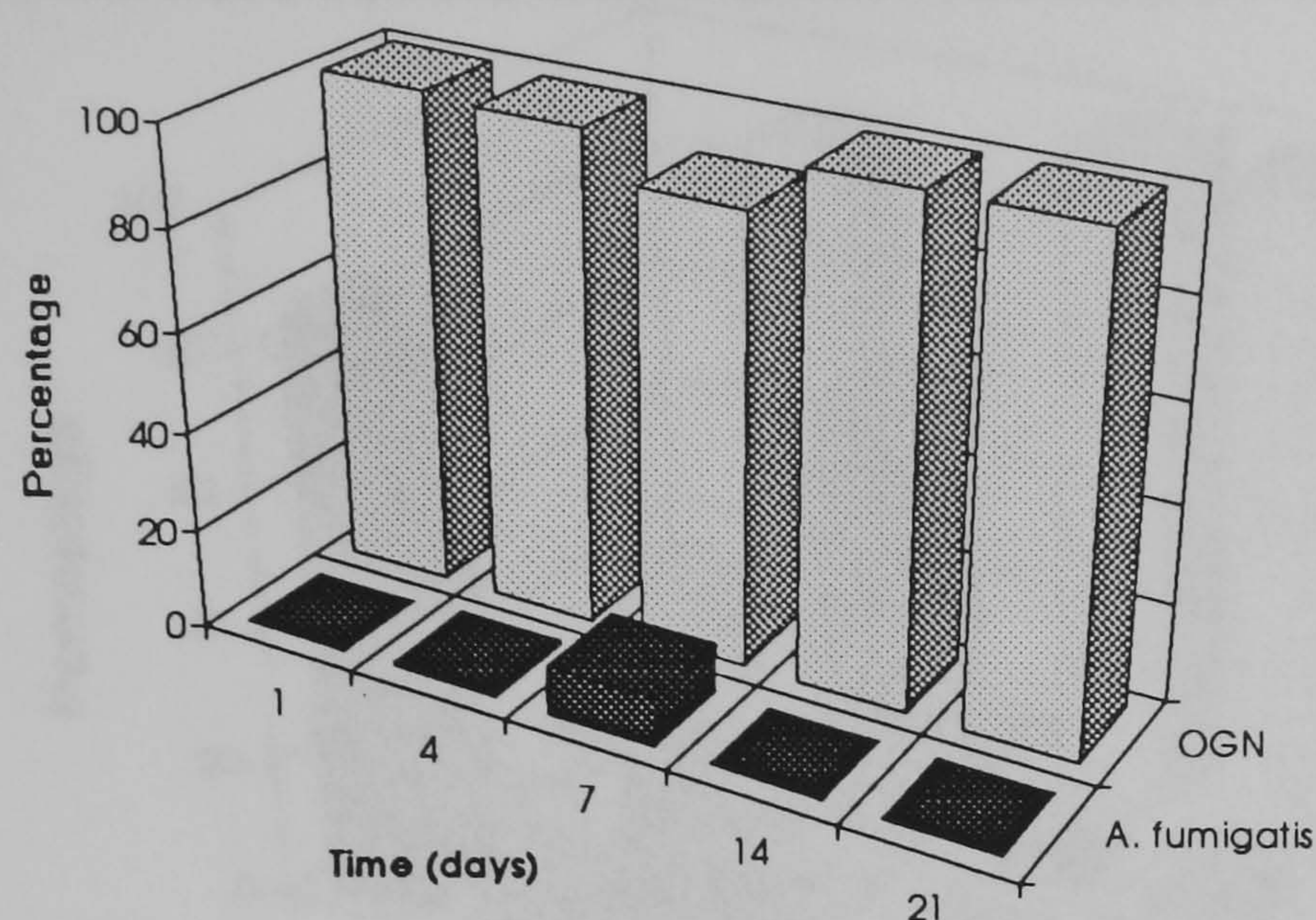


Figure 4.2 Percentage profile of microorganisms on copper at 45°C.

Table 4.1 Percentage profile of microbial species on copper at 45°C.

Species \ Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	0	0	0	0	0
<i>Methylobacterium</i> spp.	0	0	0	0	0
<i>Aspergillus fumigatus</i>	0	1	10	0	0
Gram Negative Bacteria	100	99	90	100	100

From the copper surface immersed in the culture vessel at 45°C the OGN bacteria dominated over the time course of the experiment with *A. fumigatus* being present at days 4 and 7 at 1% and 10 % respectively.

(iii) Percentage profile of microorganisms on copper in soft water at 50°C

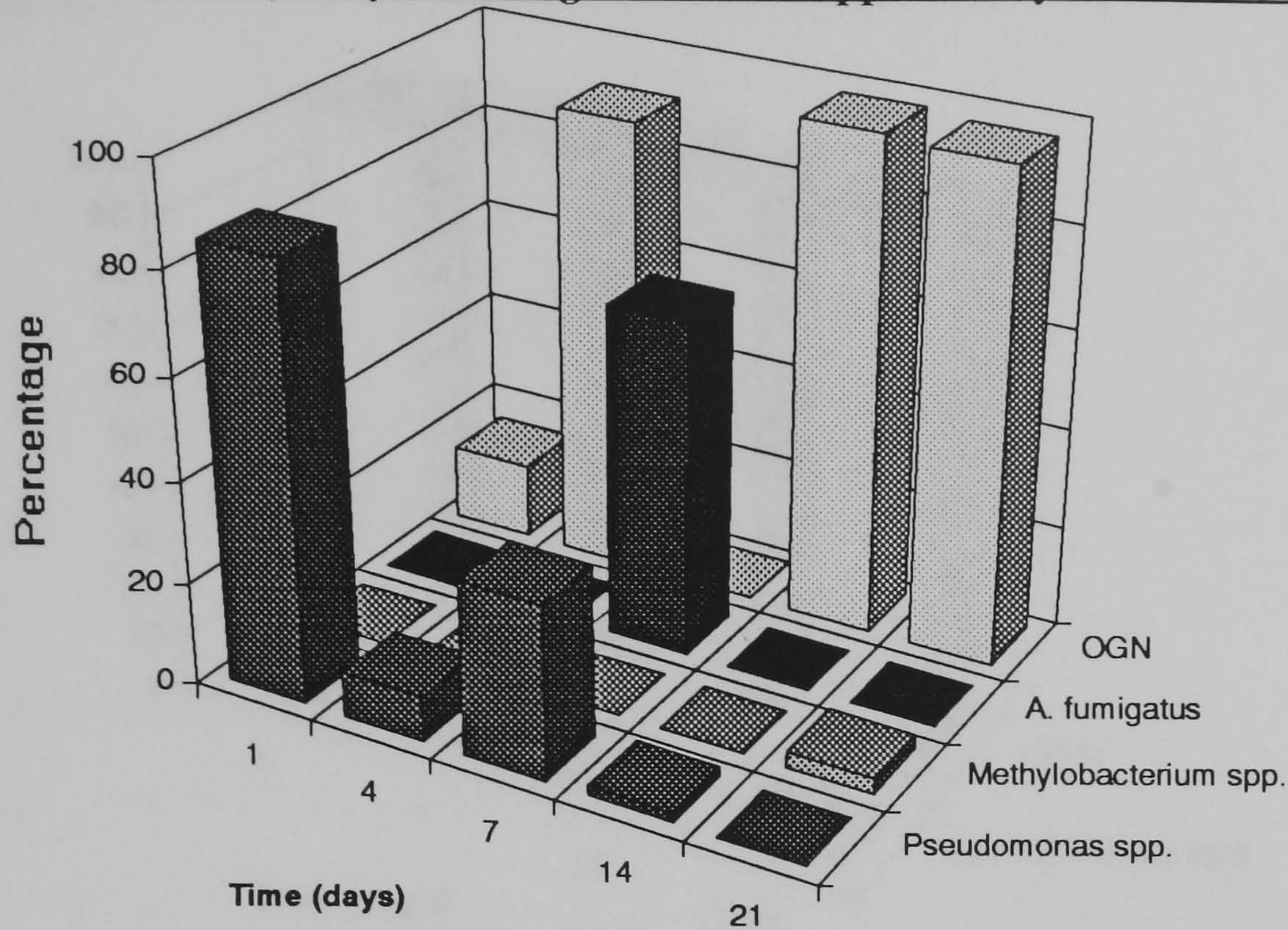


Figure 4.3 Percentage profile of microorganisms on copper at 50°C.

Table 4.2 Percentage profile of microbial species on copper at 50°C.

Species / Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	85	9	34	2	0
<i>Methylobacterium</i> spp.	0	0	0	0	3
<i>Aspergillus fumigatus</i>	0	0	66	0	0
Gram negative bacteria	15	91	0	98	97

Pseudomonas spp. were recovered as the dominant species at day 1 and declined at day 4 but they recovered at day 7 to represent 34 % of the population. They were succeeded by OGN at day 4. *A. fumigatus* only dominated at day 7 and was succeeded by the OGN that dominated at >90 % at days 14 and 21 respectively.

(iv) Percentage profile of bacteria on copper in soft water at 55°C

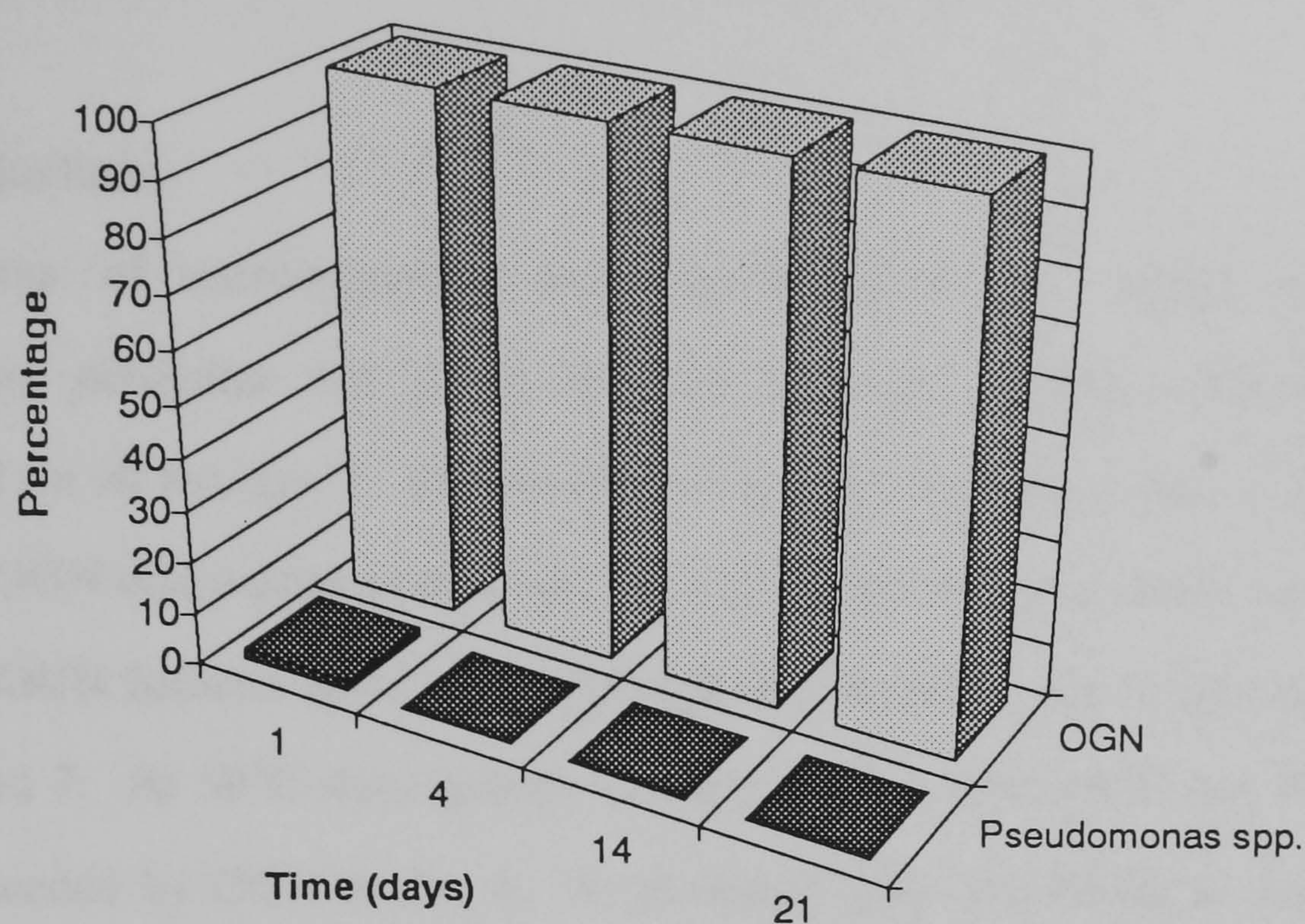


Figure 4.4 Percentage profile of bacteria on copper at 55°C.

Table 4.3 Percentage profile of microbial species on copper at 55°C.

Species / Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	2	0	0	0	0
<i>Methylobacterium</i> spp.	0	0	0	0	0
<i>Aspergillus fumigatus</i>	0	0	0	0	0
Gram Negative Bacteria	98	100	100	100	100

As the temperature was increased to 55°C only two groups of bacteria were observed with the other Gram-negative (OGN) bacteria dominating from day 1 to day 21. *Pseudomonas* spp. were present only at day 1 at less than 5% of the population and not at any time after day 1.

(v) In Summary

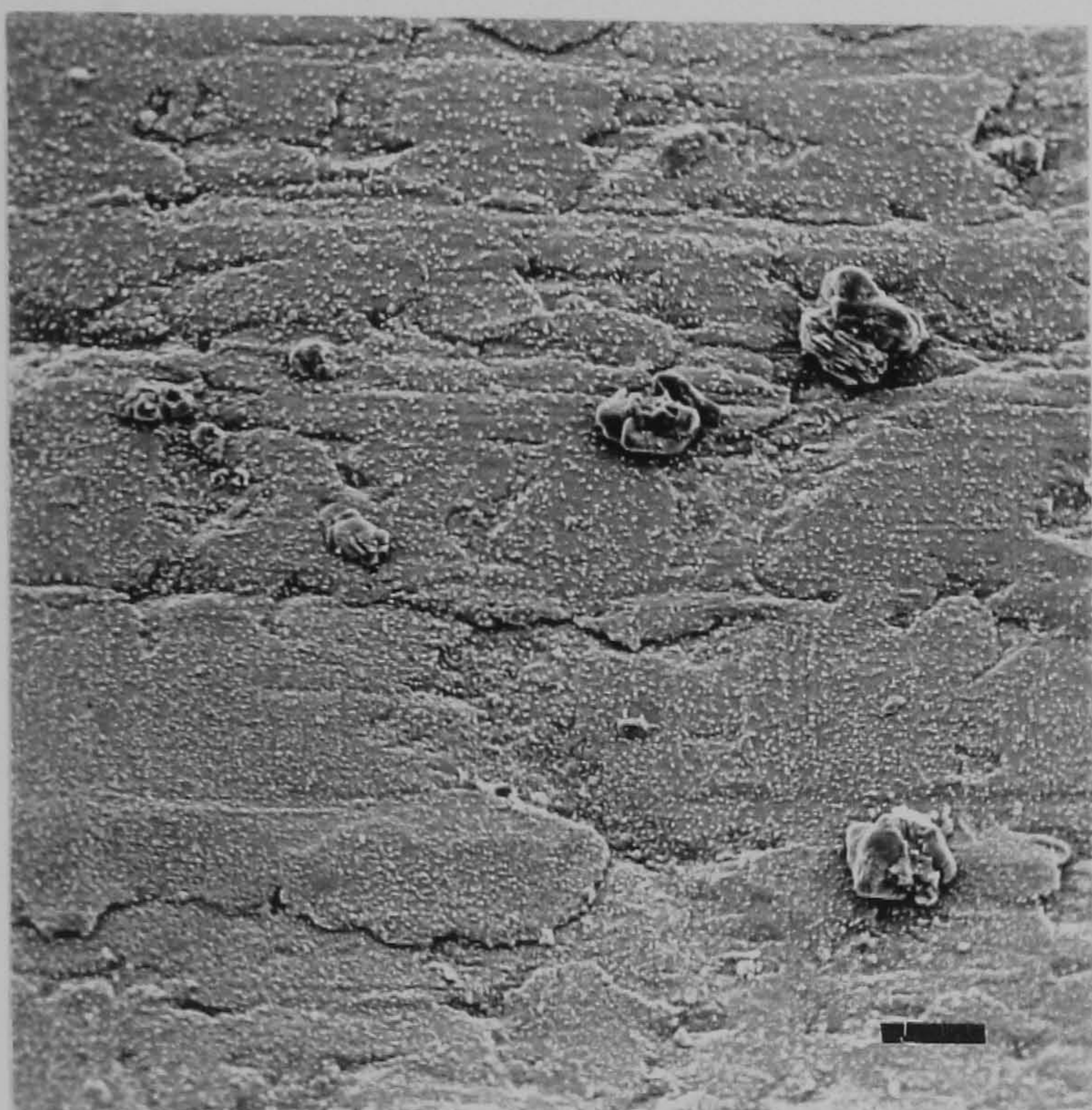
Copper Surfaces

Four groups of microorganisms were identified from the copper at 40°C with *Aspergillus fumigatus* not being detected after day 1 and *Pseudomonas* spp. recovered on all but day 7. *Methylobacterium* spp. were recovered at all but day 1 with the OGN dominating at all days. At 45°C there was a decrease to two species with the OGN bacteria dominating although *A. fumigatus* was transiently present at days 4 and 7. At 50°C three groups of bacteria were recovered with *Pseudomonas* spp. succeeded by OGN at day 4. *A. fumigatus* only dominated at day 7 and was succeeded by the OGN. As the temperature was increased to 55°C the species diversity decreased such that OGN bacteria dominated throughout with the only other species *Pseudomonas* spp. not recovered after day 1.

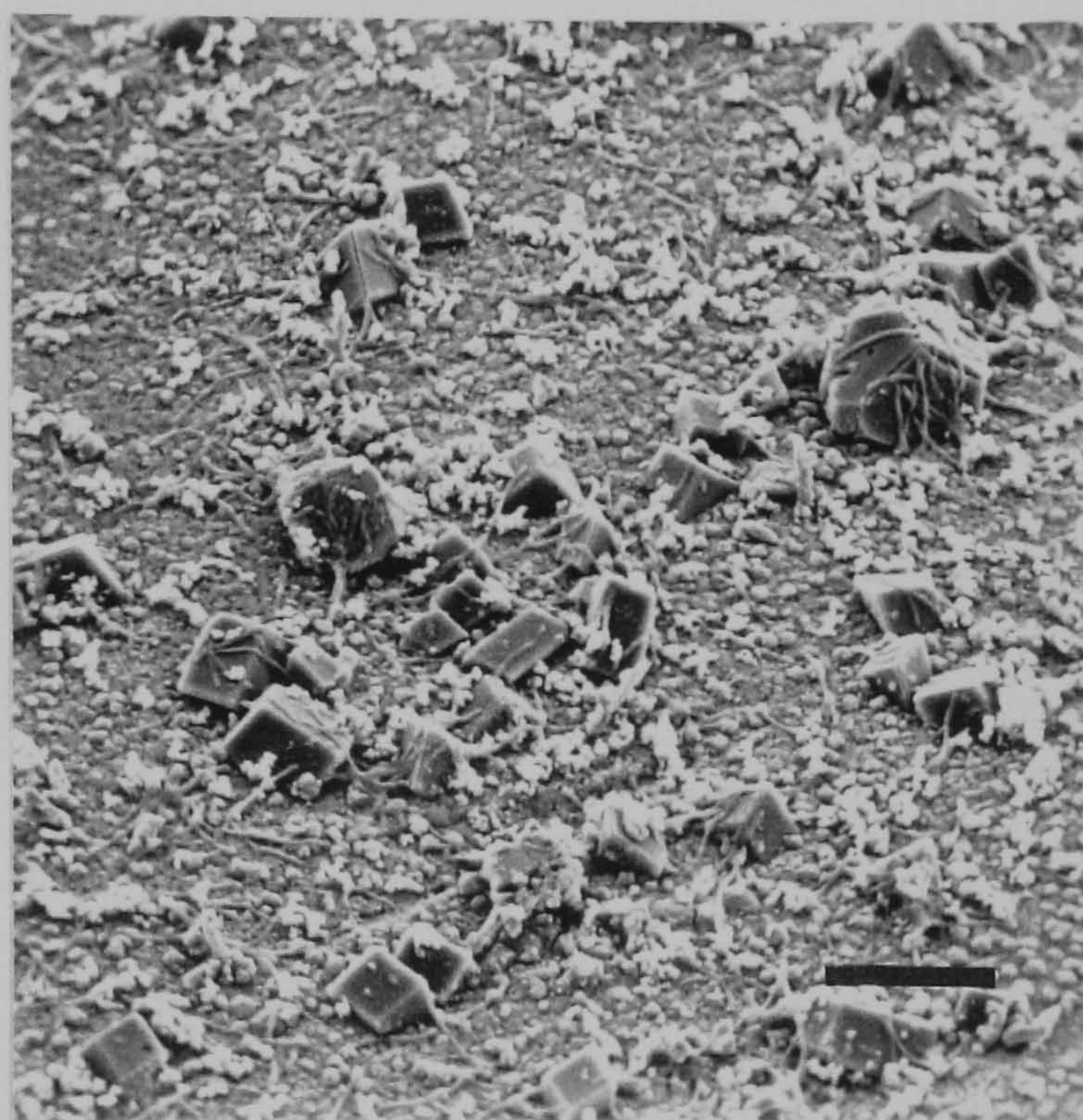
4.3.2.2 SEM's of copper coupons exposed to 40-60° C.

It was evident from the results of the scanning electron micrographs that at 40°C no bacteria could be observed at day 1 (Fig. 4.5, page 132). However, from the micrographs of 7 day coupons, 4.9 Log₁₀ bacteria cm⁻² could be observed on and around the copper carbonate crystals (Table 4.4) Debris was apparent at day 7 and is also seen at day 14, although bacteria cannot be identified in these micrographs. From coupons immersed for 21 days, bacteria can be observed readily between the particulate matter although enumeration of bacteria on the surface could not be carried out as discrimination of individual bacteria was not possible. Only individual bacteria were identifiable on coupons immersed at 45°C for up to 7 days and at days 14 and 21 the bacteria were observed to be held in a mesh like material that could not yield a bacterial count (data not shown). Similarly bacteria could not be seen on coupons from 50°C at days 1 and 7 but at days 14 and 21 there were 4.6 and 4.5 Log₁₀ bacteria cm⁻² respectively. Again at 55°C bacteria were not present on the coupons extracted from day 1 and 7 as well as day 14 (Fig. 4.6). However, 4.9 Log₁₀ bacteria cm⁻² were counted on the coupons removed after 21 days (Table 4.7). When the coupons of copper which were immersed in the vessel at 60°C were investigated no bacteria could be observed on any of the coupons.

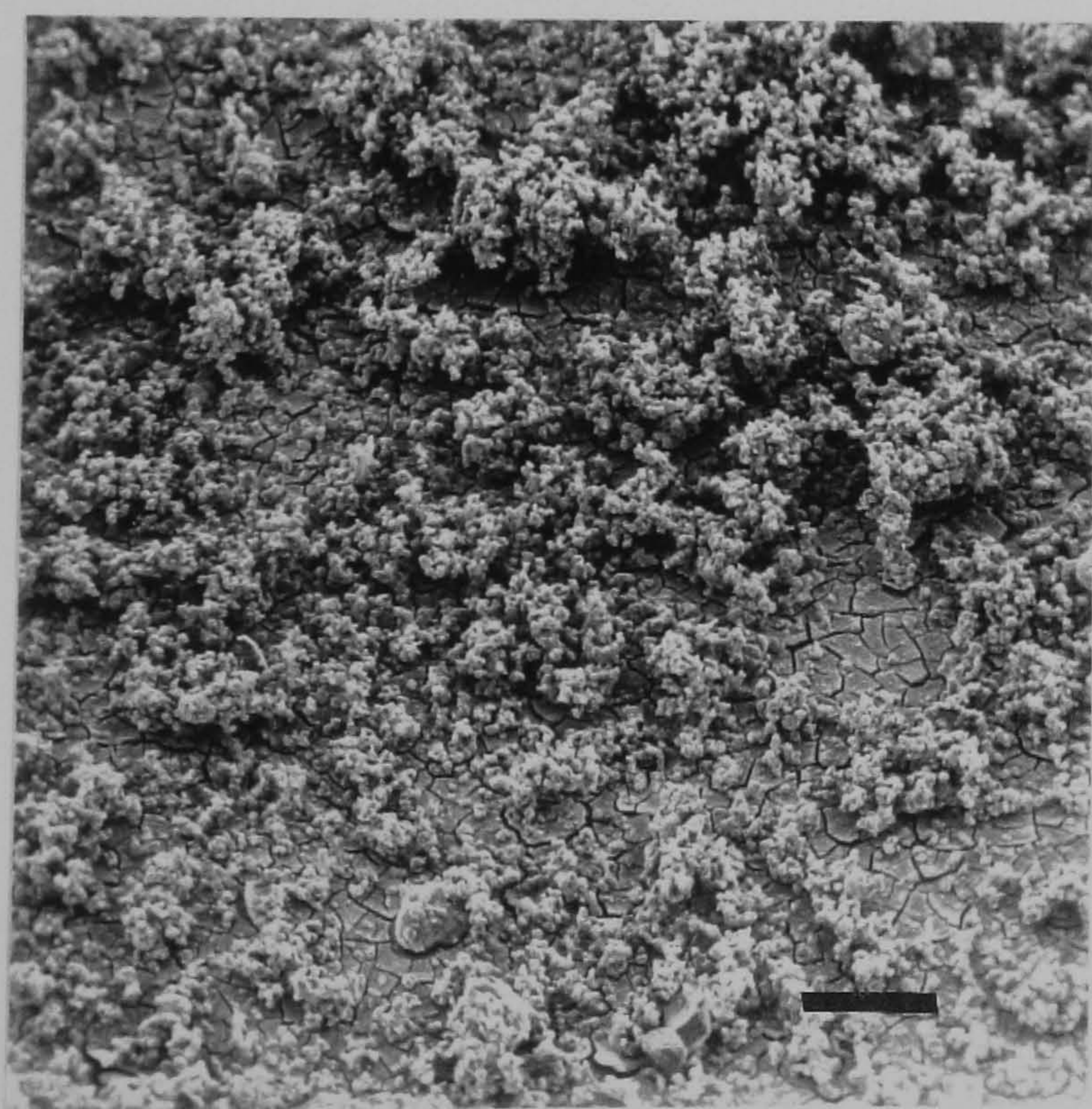
Figure 4.5 SEM of copper coupons exposed to temperatures of 40°C (Marker bar denotes 10 μm).



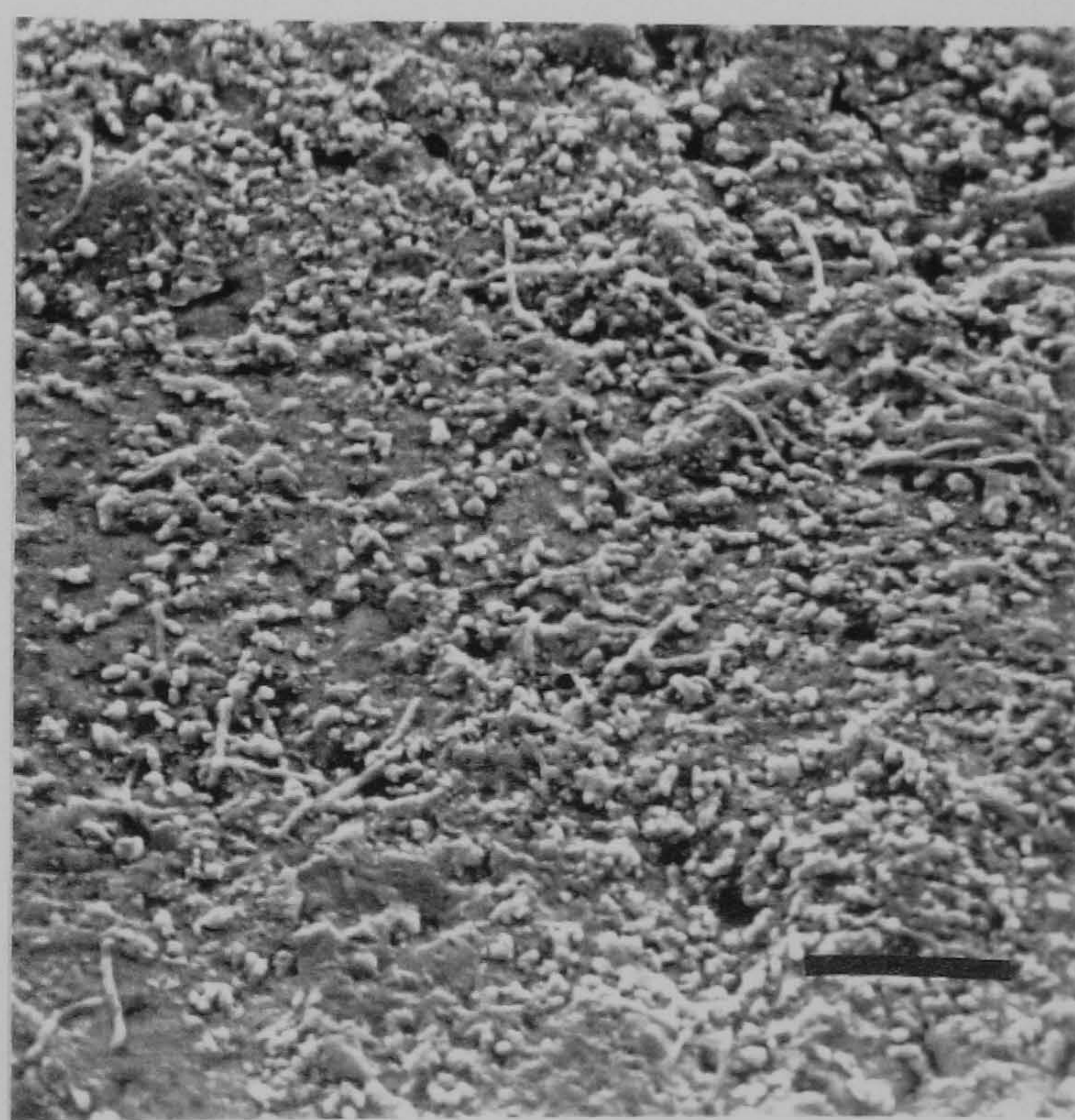
(A) 1 Day



(B) 7 Day



(C) 14 Day



(D) 21 Day

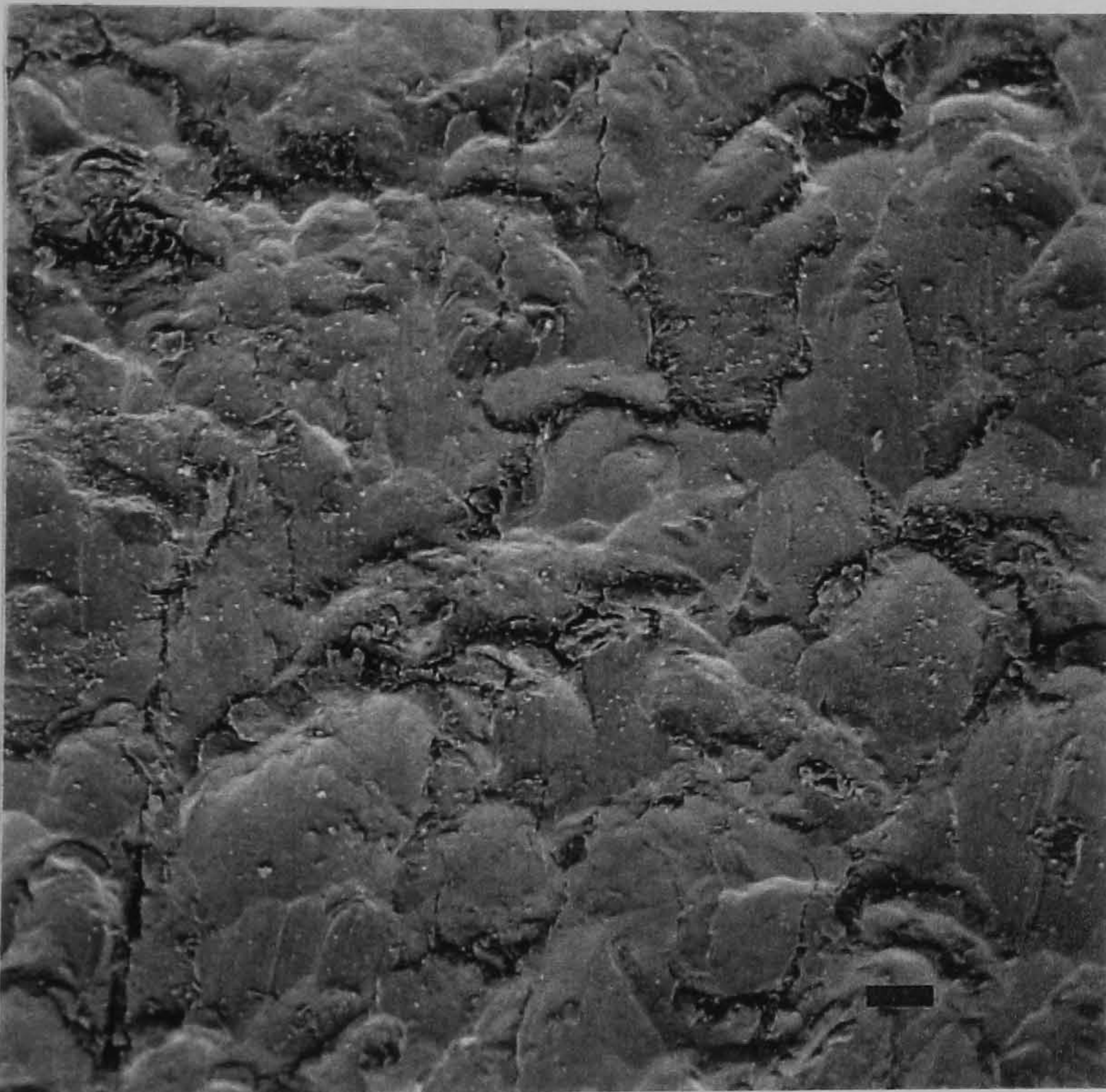
Figure 4.6 SEM of copper coupons exposed to temperatures of 55°C (Marker bar denotes 10 μm).



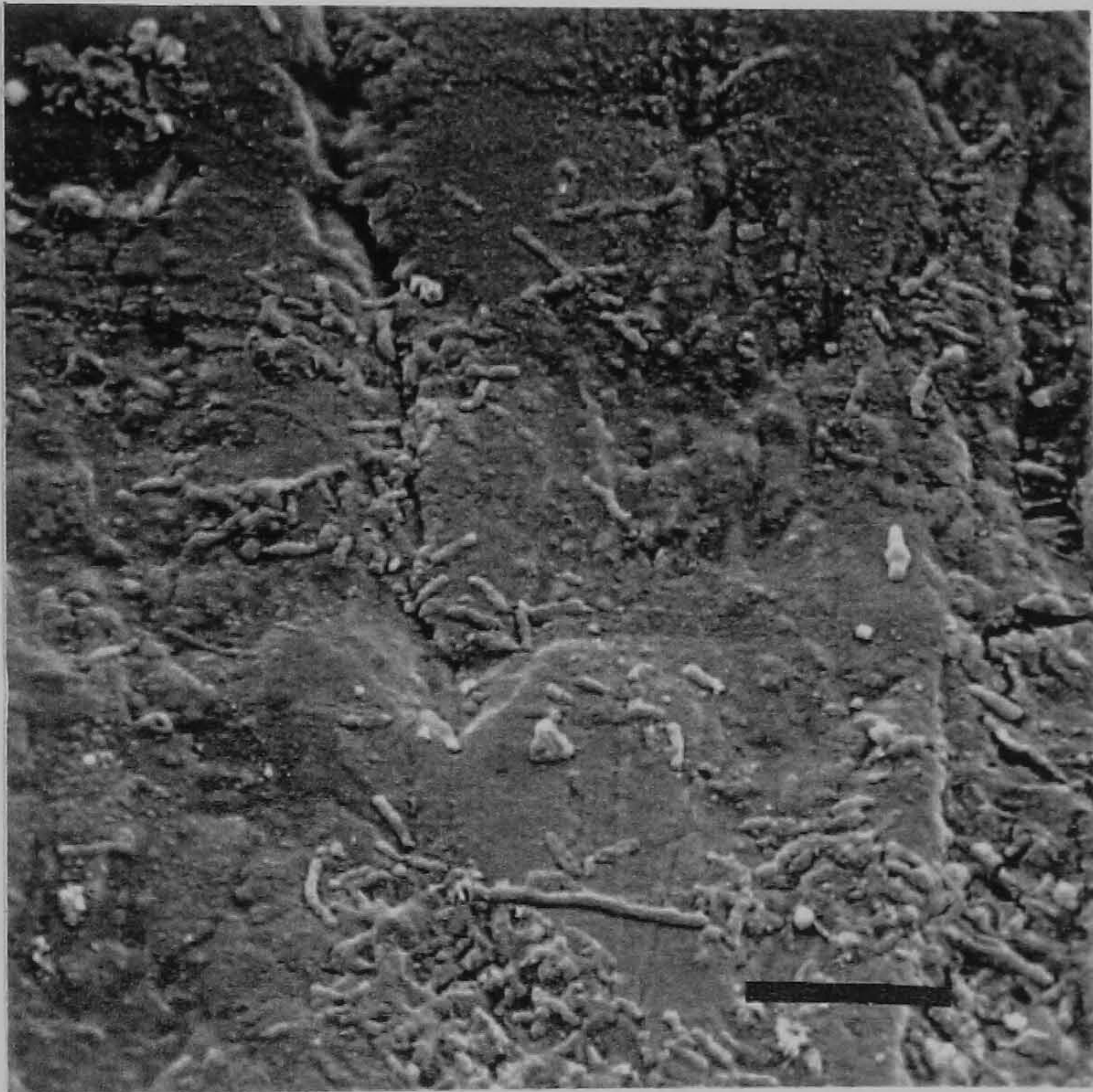
(A) 1 Day



(B) 7 Day

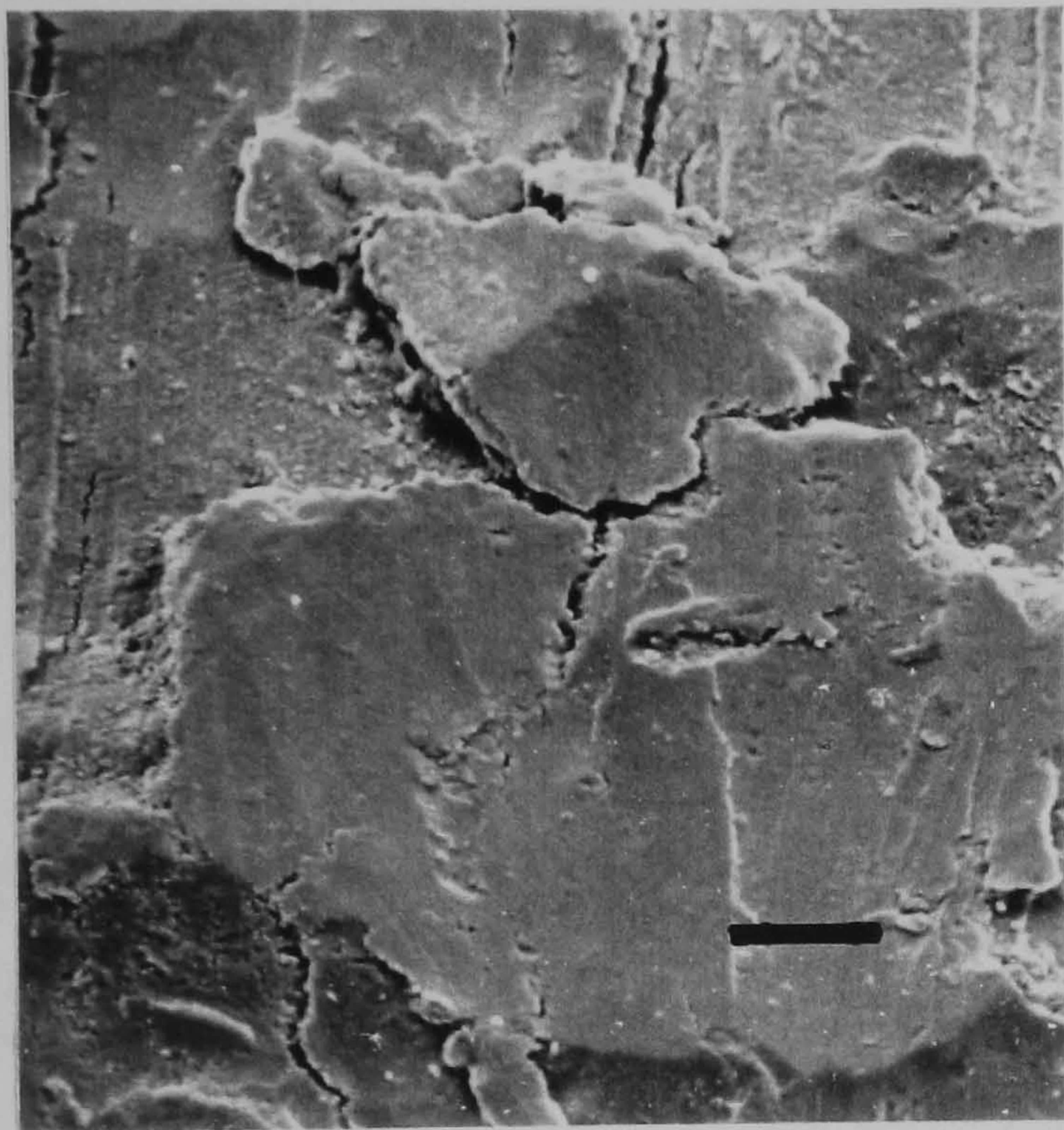


(C) 14 Day

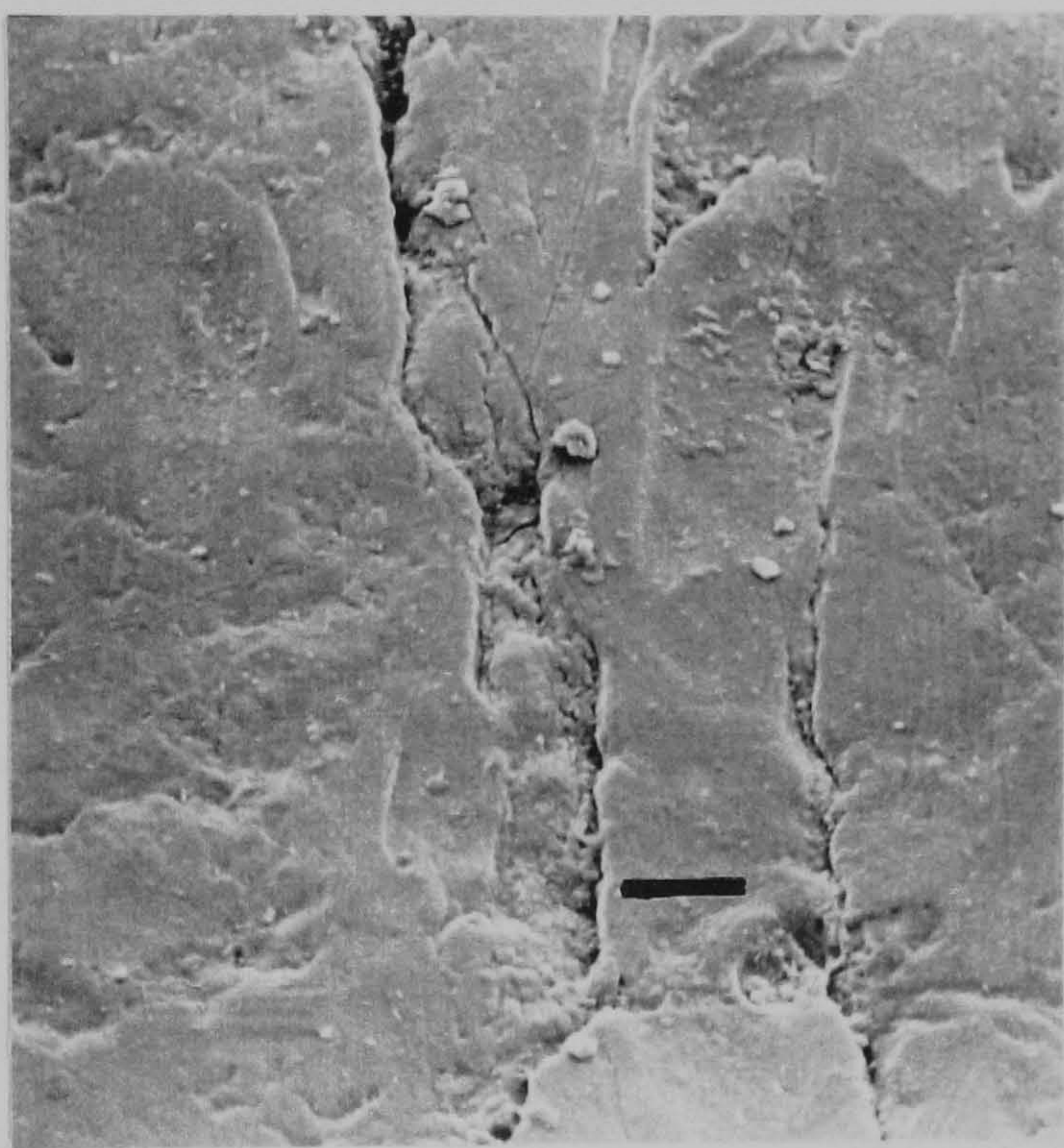


(D) 21 Day

Figure 4.7 SEM of copper coupons exposed to temperatures of 60°C (Marker bar denotes 10 μm).



(A) 1 Day



(B) 7 Day



(C) 14 Day



(D) 21 Day

Table 4.4 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on copper at 40°C

Day	Total Count	Error (±)	Log	Viable Count (Log)
1	0	-	-	2.5
7	94500	22050	4.9	2.8
14	0	-	-	2.2
21	nc	-	-	2.9

nc denotes no count

Table 4.5 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on copper at 45°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	0	-	-	3.7
7	0	-	-	3.8
14	nc	-	-	3.6
21	nc	-	-	3.7

nc denotes no count

Table 4.6 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on copper at 50°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	0	-	-	2.5
7	0	-	-	2.8
14	54990	6110	4.7	2.4
21	nc	-	-	3.43

nc denotes no count

Table 4.7 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on copper at 55°C

Day	Total Count	Error	Log	Viable Count (Log)
1	0	-	-	4.9
7	0	-	-	4.7
14	0	-	-	4.85
21	83600	21660	4.92	4.8

nc denotes no count

4.3.3 Biofilm development of glass surfaces

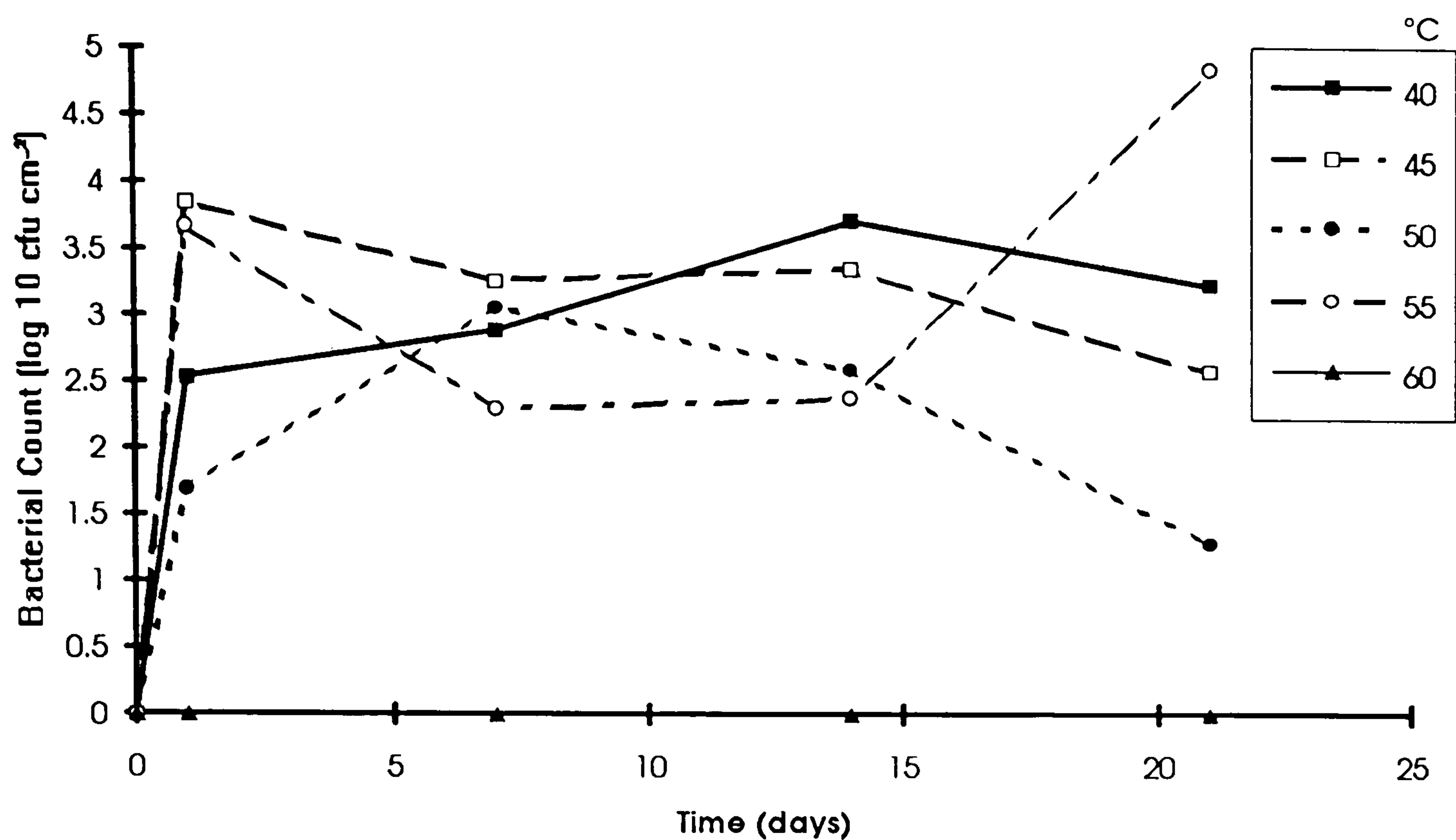


Figure 4.8 Colonisation of glass in the presence of copper.

From the biofilms examined at day 1 the lowest recovery of bacteria was at 50°C at 1.7 Log₁₀ cfu cm⁻² with a maximum at 45°C of 3.8 Log₁₀ cfu cm⁻² bacteria. At day 7 the recovery ranged from 2.3 Log₁₀ cfu cm⁻² at 55°C to 3.4 Log₁₀ cfu cm⁻². A similar range of bacteria were recovered at day 14, 2.0 - 3.4 Log₁₀ cfu cm⁻², except at 55°C where 4.8 Log₁₀ cfu cm⁻² bacteria were recovered. The range of viable bacteria recovered at day 21 ranged from 1.5 Log₁₀ cfu cm⁻² at 50°C to 2.8 & 3.4 Log₁₀ cfu cm⁻² bacteria being recovered at 45°C & 40°C respectively. At 55°C, 4.8 Log₁₀ cfu cm⁻² were recovered. In comparison no bacteria were obtained from coupons immersed in the vessel at 60°C. There were no statistical differences obtained between 40 & 45°C and 45 & 55°C. In comparison the differences between 40 & 50°C, 45 & 50°C and 50 & 55°C are statistical different ($P = 0.046$, $P = 0.027$ and $P = 0.046$) respectively.

4.3.3.1 Profiles of the microbial types recovered from glass coupons (40-60° C).

(i) Percentage profile of microorganisms on glass at 40°C

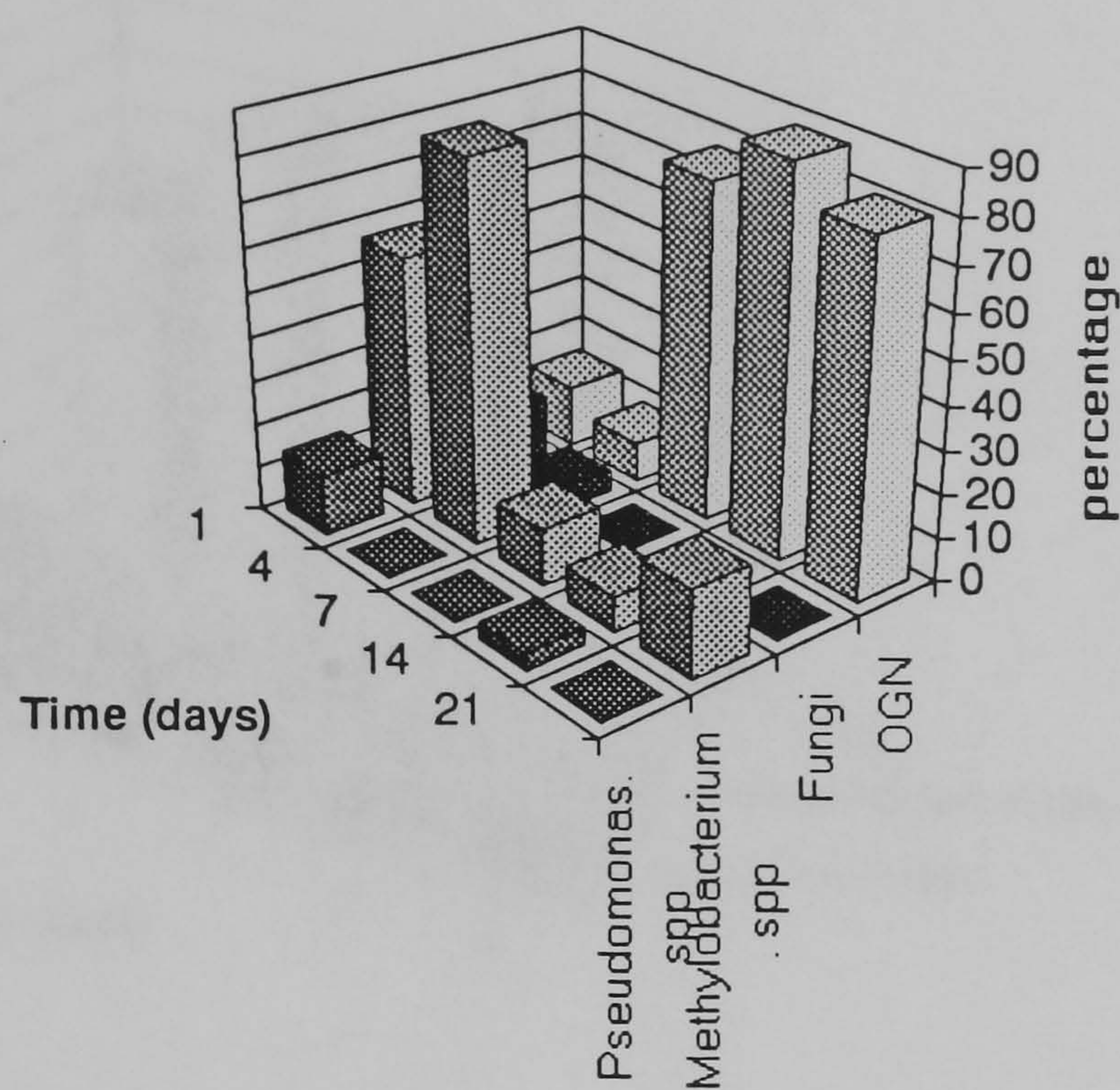


Figure 4.9 Percentage profile of microorganisms on glass at 40°C.

Table 4.8 Percentage profile of microbial species on glass at 40°C.

Species / Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	12	0	0	4	0
<i>Methylobacterium</i> spp.	64	86	13	8	20
<i>Aspergillus fumigatus</i>	12	5	0	0	0
Gram negative bacteria	12	9	77	88	80

Pseudomonas spp. were recovered on day 1 at 12% and at 4% at day 14. *A. fumigatus* was only recovered at days 1 & 4 at 12% and 5% respectively. *Methylobacterium* spp. dominated at days 1 & 7 at 64% & 86% but were only recovered at less than 20% up to day 21. OGN were only recovered at 12% & 9% at day 1 & 7 before dominating the population at > 80% for the remainder of the experiment.

(ii) Percentage profile of bacteria on glass in soft water at 45°C.

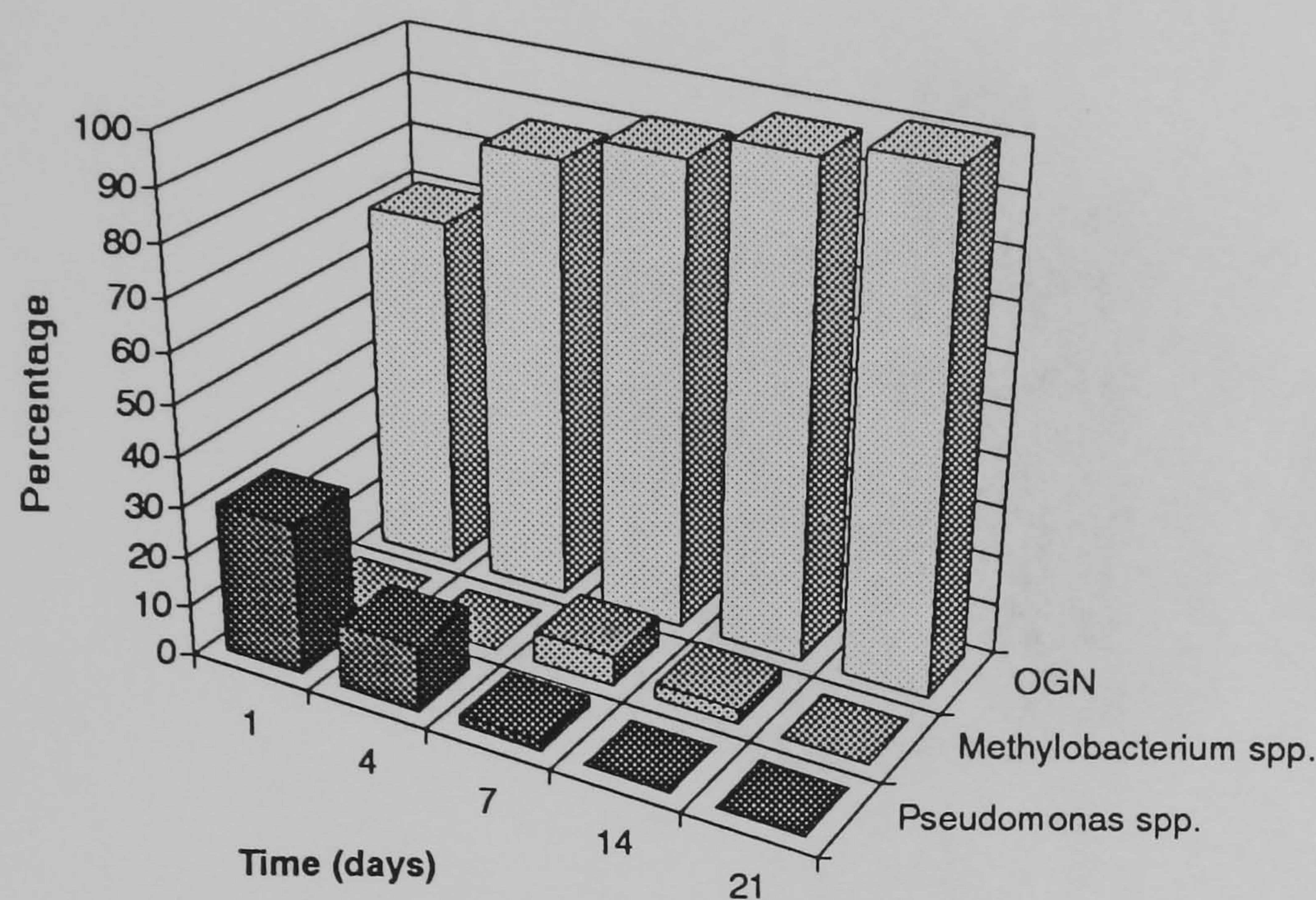


Figure 4.10 Percentage profile of bacteria on glass at 45°C.

Table 4.9 Percentage profile of bacterial species on glass at 45°C.

Species / Days	1	4	7	14	21
<i>Pseudomonas</i> spp.	30	13	2	0	0
<i>Methylobacterium</i> spp.	0	0	6	3	0
Gram negative bacteria	70	87	92	97	100

Recovery of bacteria from glass at 45°C was dominated by OGN from day 1 to day 21. *Pseudomonas* spp. were present on day 1 at 30 % but steadily declined as part of the flora until day 14 when they were not detected. *Methylobacterium* spp. were only present in detectable numbers at days 7 and 14 but represented less than 10 % of the total population.

(iii) Percentage profile of microorganisms on glass at 50°C in soft water.

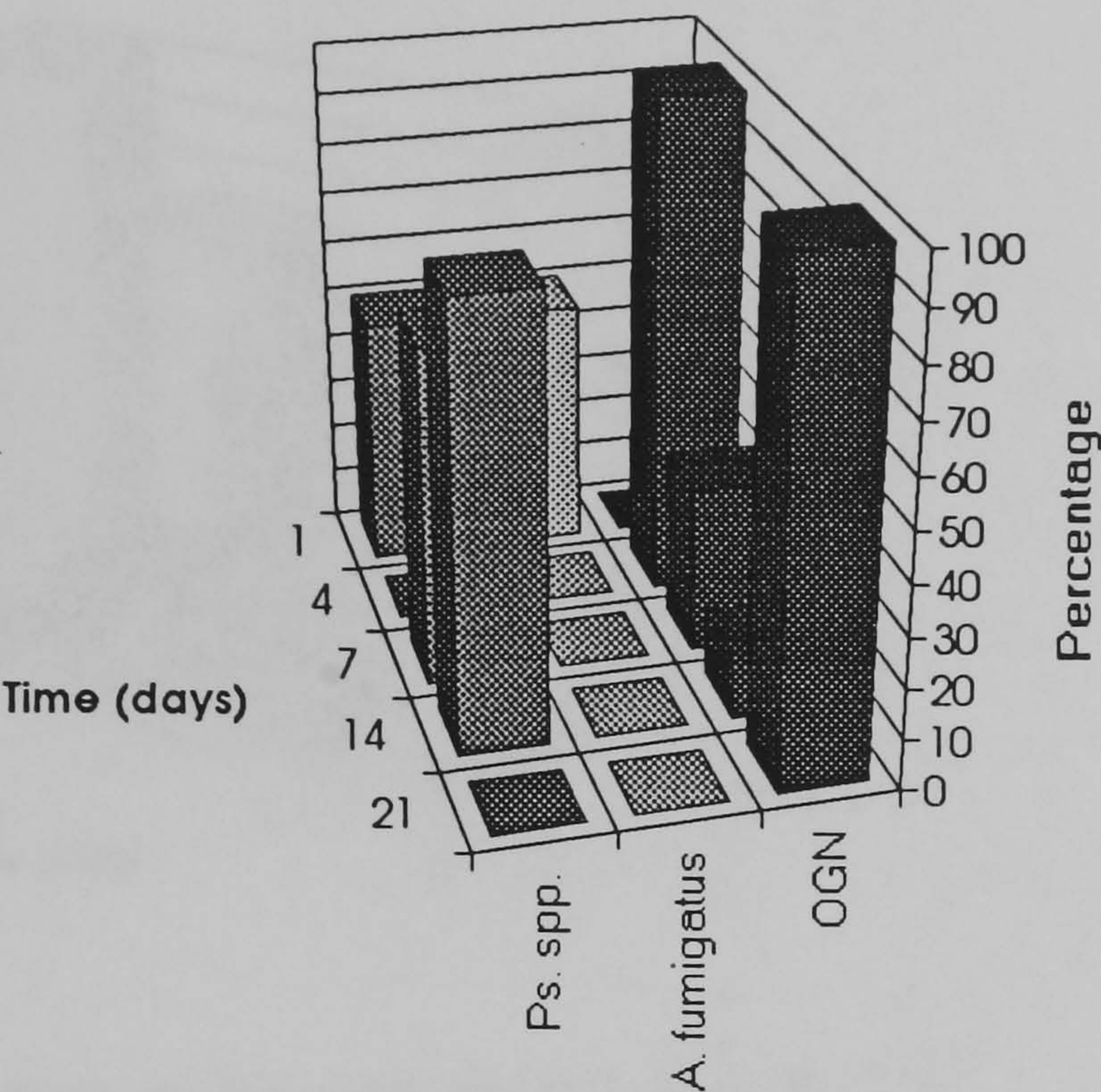


Figure 4.11 Percentage profile of microorganisms on glass at 50°C.

Table 4.10 Percentage profile of microbial species on glass at 50°C.

Species / Day	1	4	7	14	21
<i>Pseudomonas</i> spp.	50	0	67	87	87
<i>Aspergillus fumigatus</i>	50	0	0	0	0
Gram negative bacteria	0	100	33	13	13

At this temperature *Pseudomonas* spp. and *A. fumigatus* were recovered in equal numbers at day 1 but only OGN were detected at day 4. At day 14 *Pseudomonas* spp. dominated over OGN which were the only bacteria present at day 21.

(iv) Percentage profile of bacteria on glass at 55°C in soft water.

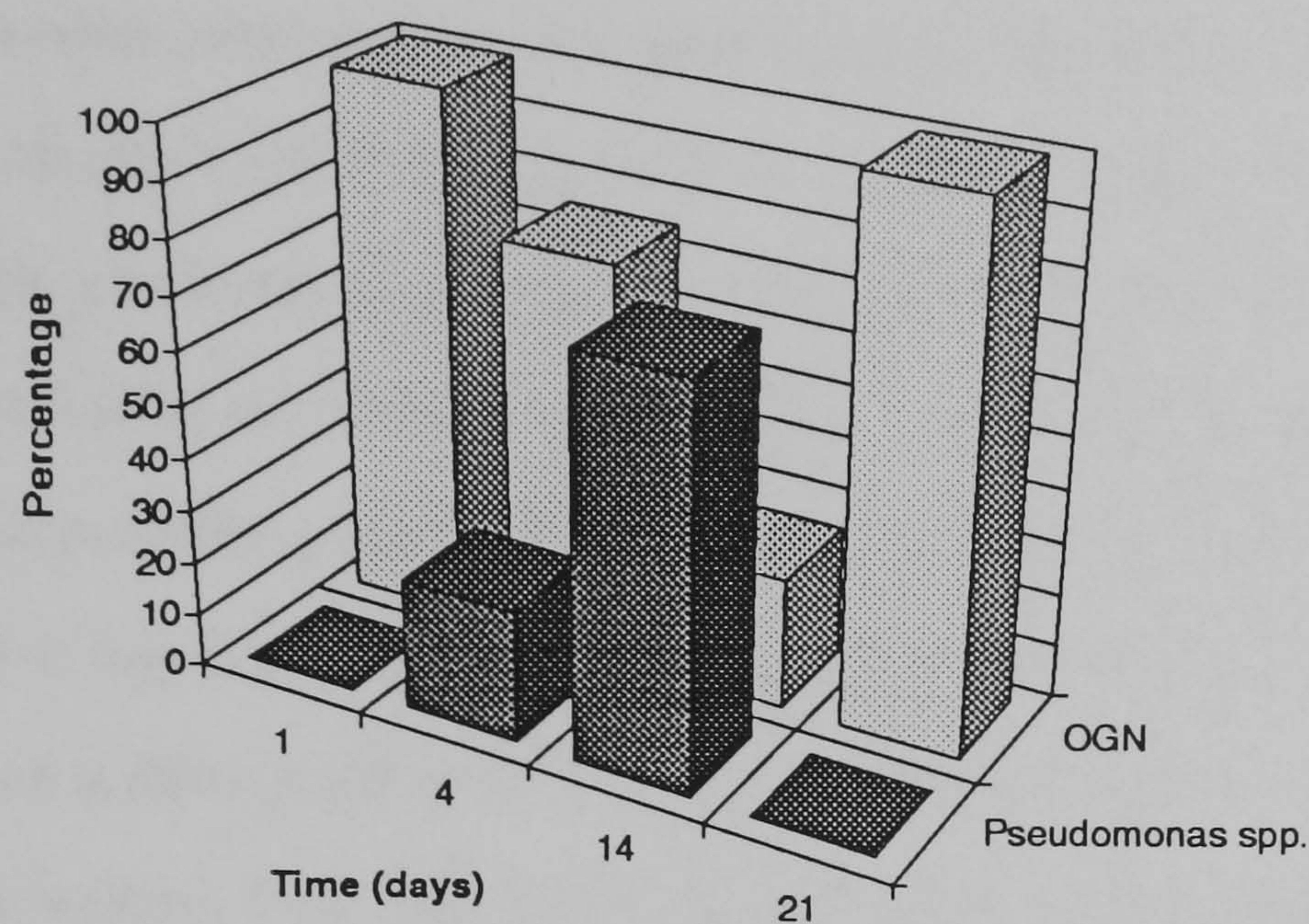


Figure 4.12 Percentage profile of bacteria on glass at 55°C.

Table 4.11 Percentage profile of bacterial species on glass at 55°C.

Species / Day	1	7	14	21
<i>Pseudomonas</i> spp.	0	25	75	0
<i>Aspergillus fumigatus</i>	0	0	0	0
Gram negative bacteria	100	75	25	100

The OGN dominated at all but day 14 where they were succeeded by the *Pseudomonas* spp. which were only present at day 4 and day 14.

4.3.3.2 SEM of glass coupons exposed to 40-60° C.

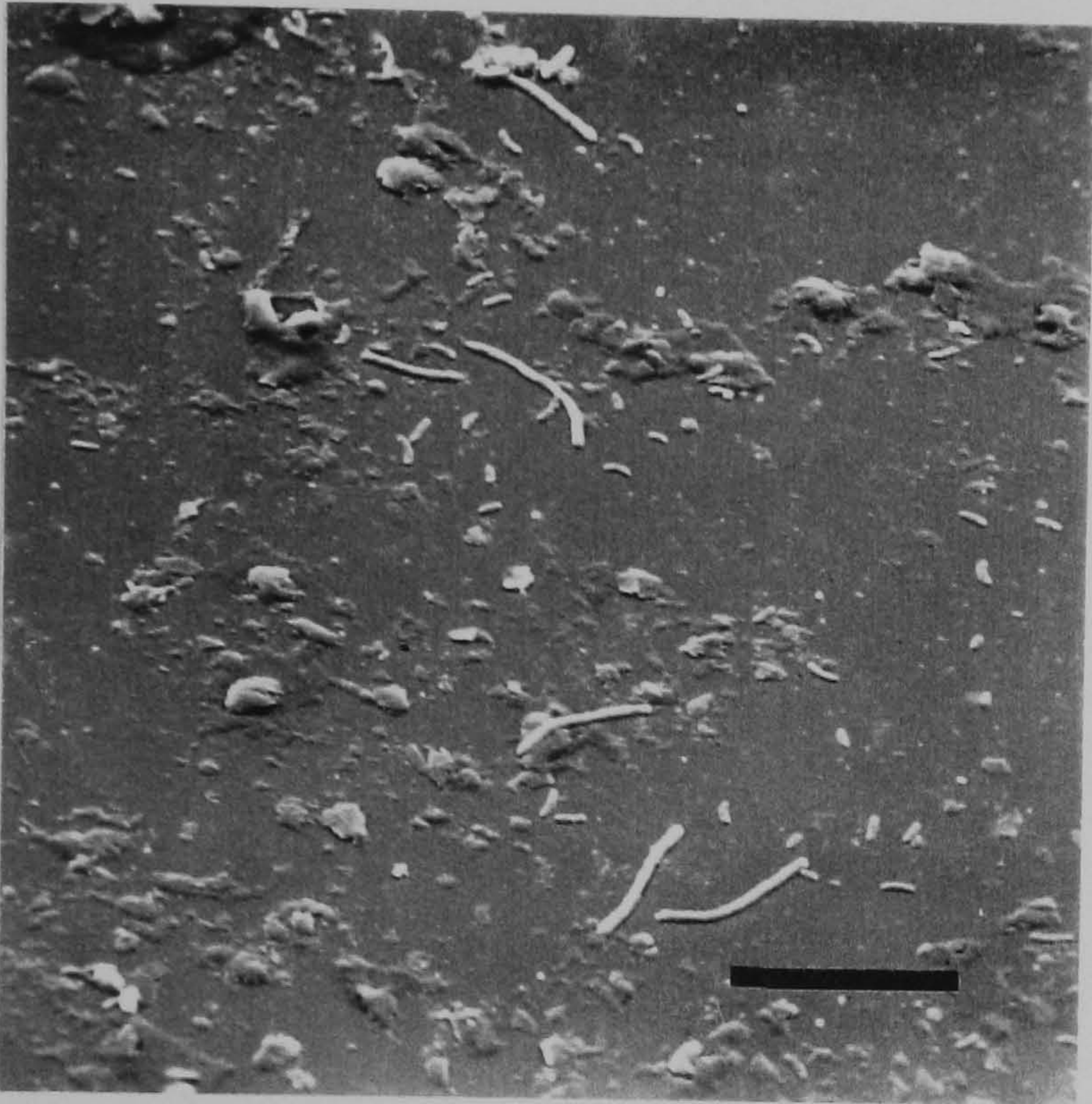
Bacteria were observed on the glass coupons extracted from the culture vessel at 40°C from day 1 (4.77 Log₁₀ bacteria cm⁻²) to day 21 (4.70 Log₁₀ bacteria cm⁻²). Although not shown, similar profiles were observed at 45°C and 50°C. At 45°C, 4.61 Log₁₀ bacteria cm⁻² were counted at day 1 and 4.19 Log₁₀ bacteria cm⁻² at day 21. From the coupons immersed in the culture at 50°C, 3.45 Log₁₀ bacteria cm⁻² were counted at day 1 and 4.51 Log₁₀ bacteria cm⁻² at day 21. At 55°C there appears to have been a slow build up with only a few bacteria spread over the surface at day 1, which correlates with only 3.64 Log₁₀ bacteria cm⁻² counted on the coupons. The number of bacteria enumerated from the micrographs was 4.49 and 4.74 Log₁₀ bacteria cm⁻² at days 7 and 14 respectively. At day 21 a microcolony can be observed over the surface with the number in individual bacteria counted remaining high at 4.72 Log₁₀ bacteria cm⁻². Particulate matter can be seen on the glass coupons which had been immersed in the vessel at 60°C. Indeed one or two bacteria can be seen on the coupons extracted at day 7.

Glass Surfaces

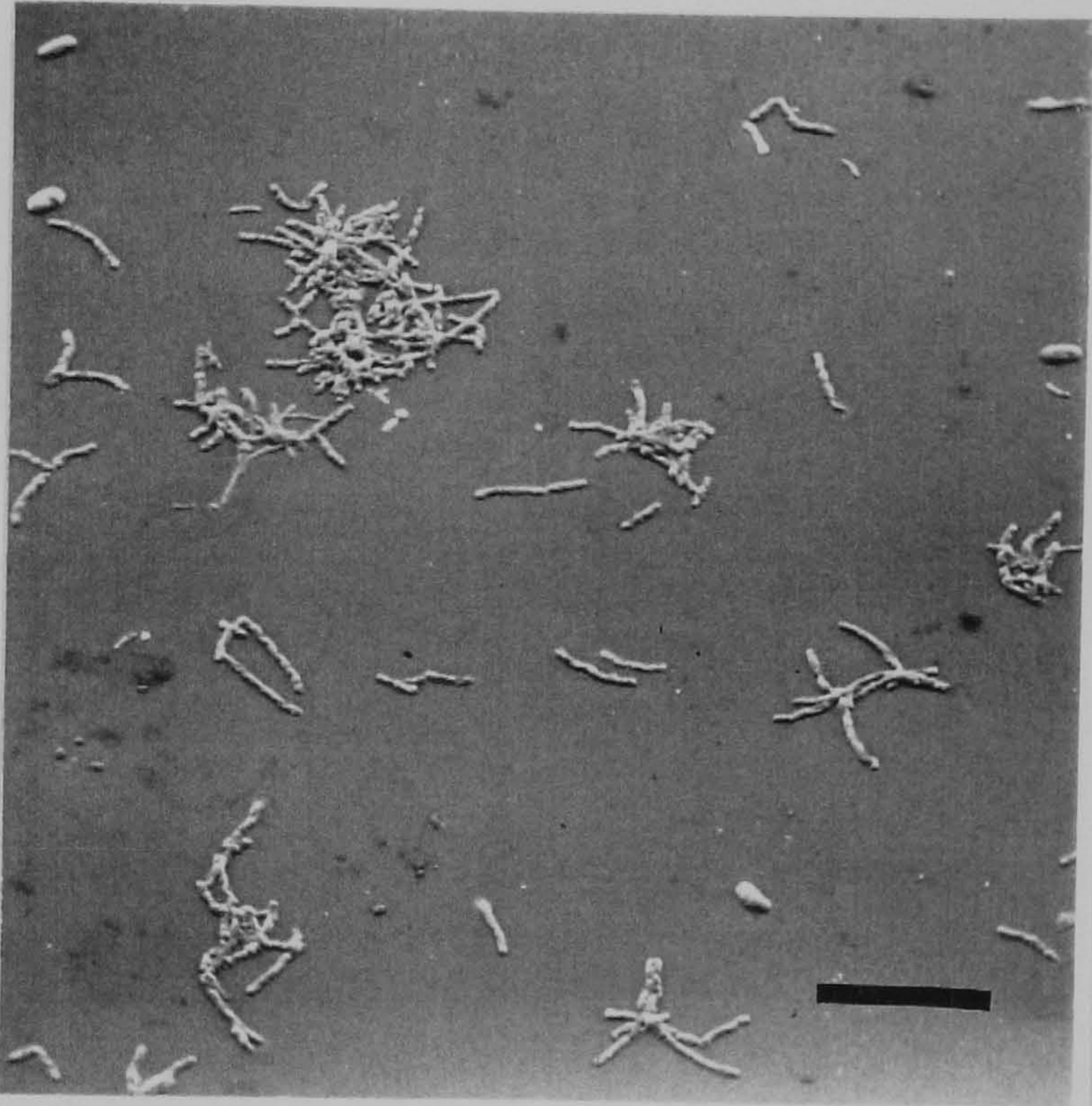
As in the population profiles from the copper surfaces the same four groups of microorganisms were identified from the glass coupons at 40°C. On the glass coupons *Methylobacterium* spp. dominated at day 1 and 4 but were succeeded by the OGN which dominated thereafter. From glass coupons at 45°C the *Methylobacterium* spp. were transiently present at days 7 and 14. *Pseudomonas* spp. were initially present but did not form part of the consortia after day 7. The OGN dominated over the time course. At 50°C *A. fumigatus* was present at day 1 but was not recovered after this. The *Pseudomonas* spp. and the OGN exhibited transient dominance with the OGN succeeding at day 21. As with the copper surface at 55°C there were only two species recovered from the glass coupons at 55C, *Pseudomonas* spp. and OGN, the latter of which dominated.

At 40°C the four bacterial groups were present on both copper and glass surfaces. *A. fumigatus* and OGN dominated at day 1 on copper but on glass the *Methylobacterium* spp. dominated at day 1 and 4. Although the *Pseudomonas* spp., *Methylobacterium* spp. and *A. fumigatus* were present the OGN dominated until day 21. *A. fumigatus* was initially present at day 1 (copper) and at days 1 and 4 on glass but not thereafter. *Pseudomonas* spp. was transiently present on both surfaces and apart from day 1 (copper) *Methylobacterium* spp. were also present as part of the population. On copper at 45°C the species diversity decreased with *A. fumigatus* only recovered at day 7 with OGN dominating from day 7 to day 21. On both copper and glass the species diversity increased again as the temperature was increased to 50°C. On copper the *Pseudomonas* spp. dominated at days 1, 7, and 14 (with *A. fumigatus* on glass at day 1). At days 4 and 21 the OGN dominated on both copper and glass. At 55°C the species diversity decreased again with only *Pseudomonas* spp. and OGN bacteria being recovered. On copper the OGN dominated from day 1 to 21 and on glass the *Pseudomonas* spp. only dominated at day 14.

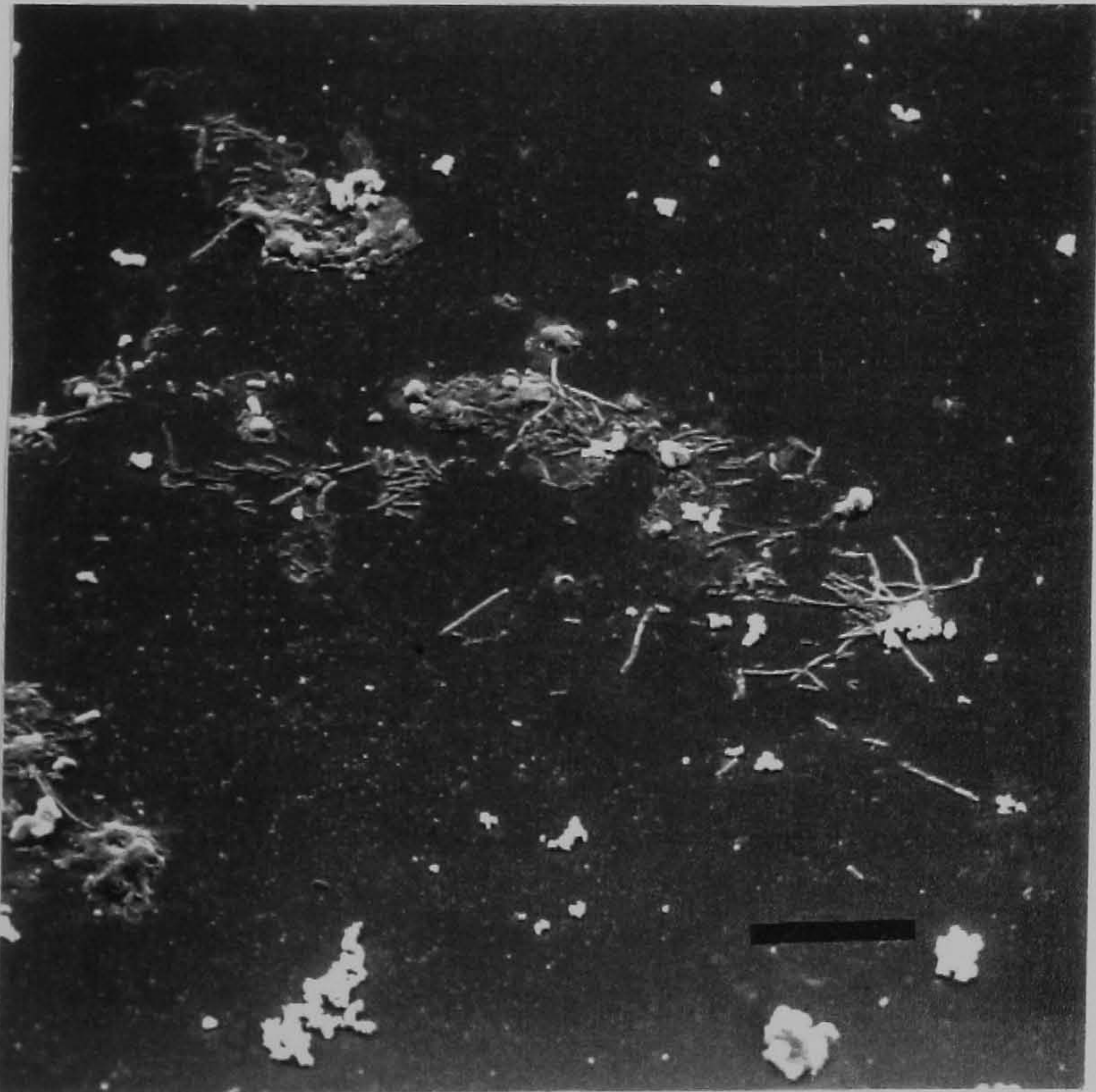
Figure 4.13 SEM of glass coupons exposed to temperatures of 40°C (Marker bar denotes 10 μm).



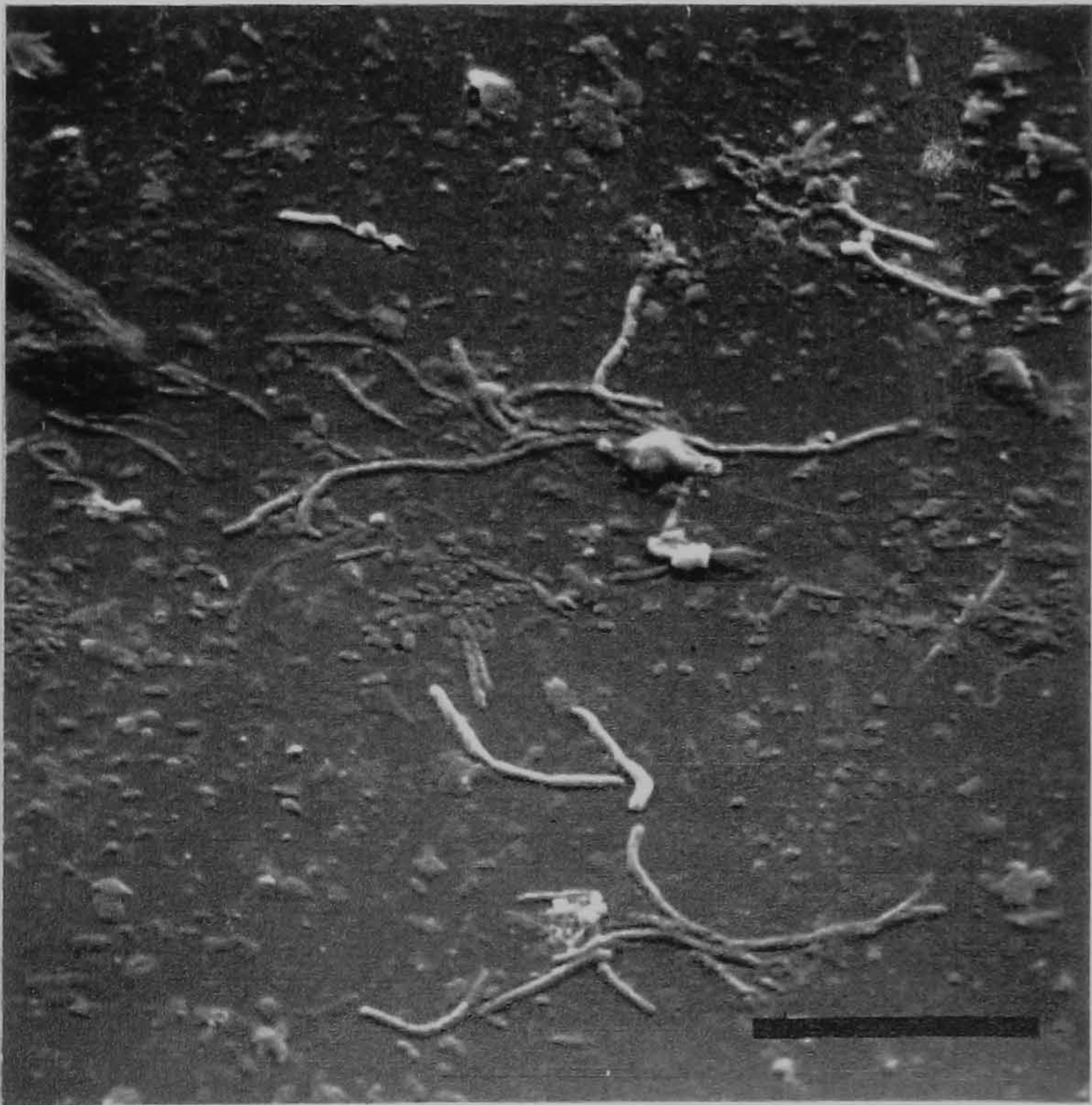
(A) 1 Day



(B) 7 Day

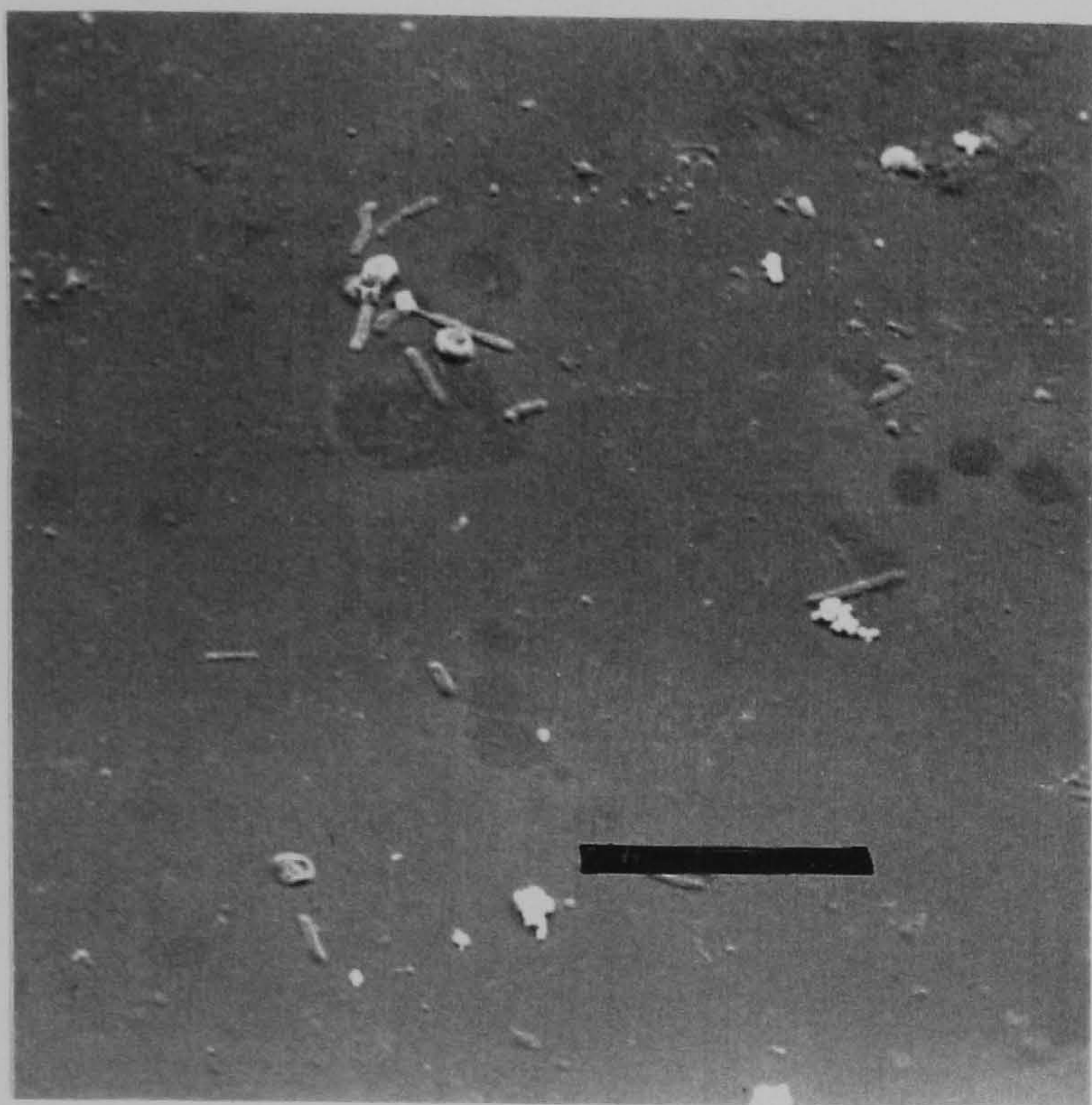


(C) 14 Day

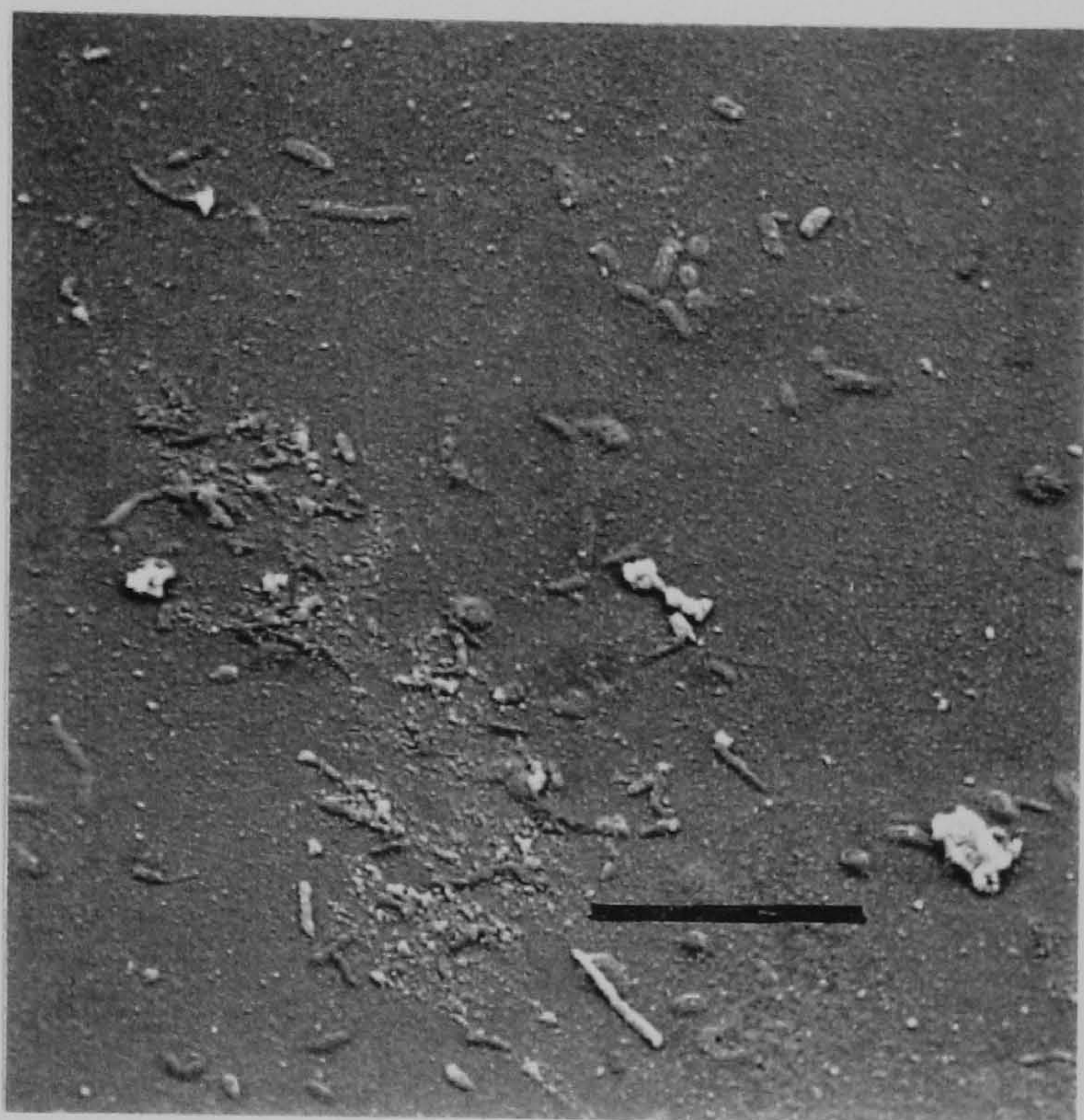


(D) 21 Day

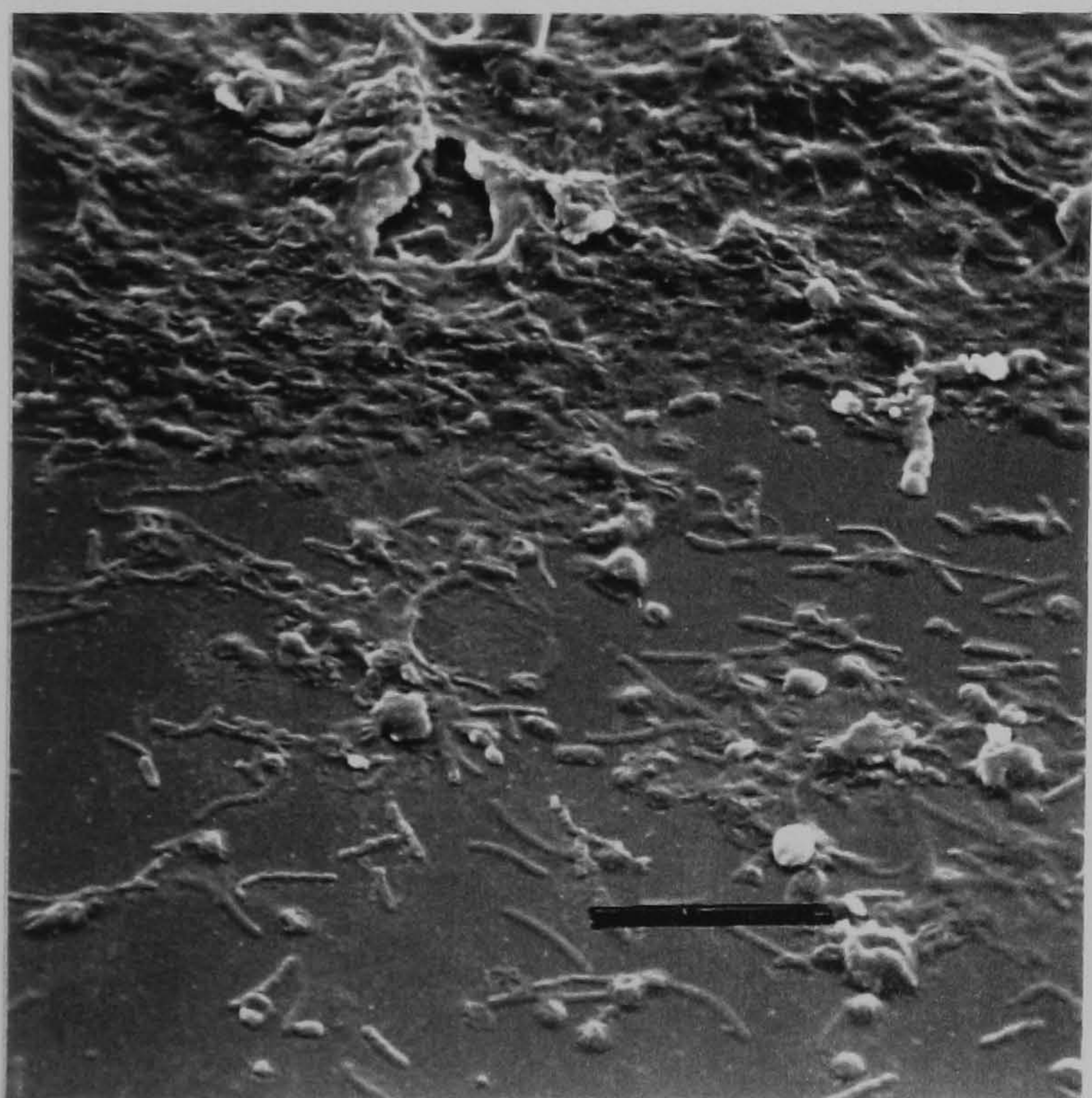
Figure 4.14 SEM of glass coupons exposed to temperatures of 55°C (Marker bar denotes 10 μm).



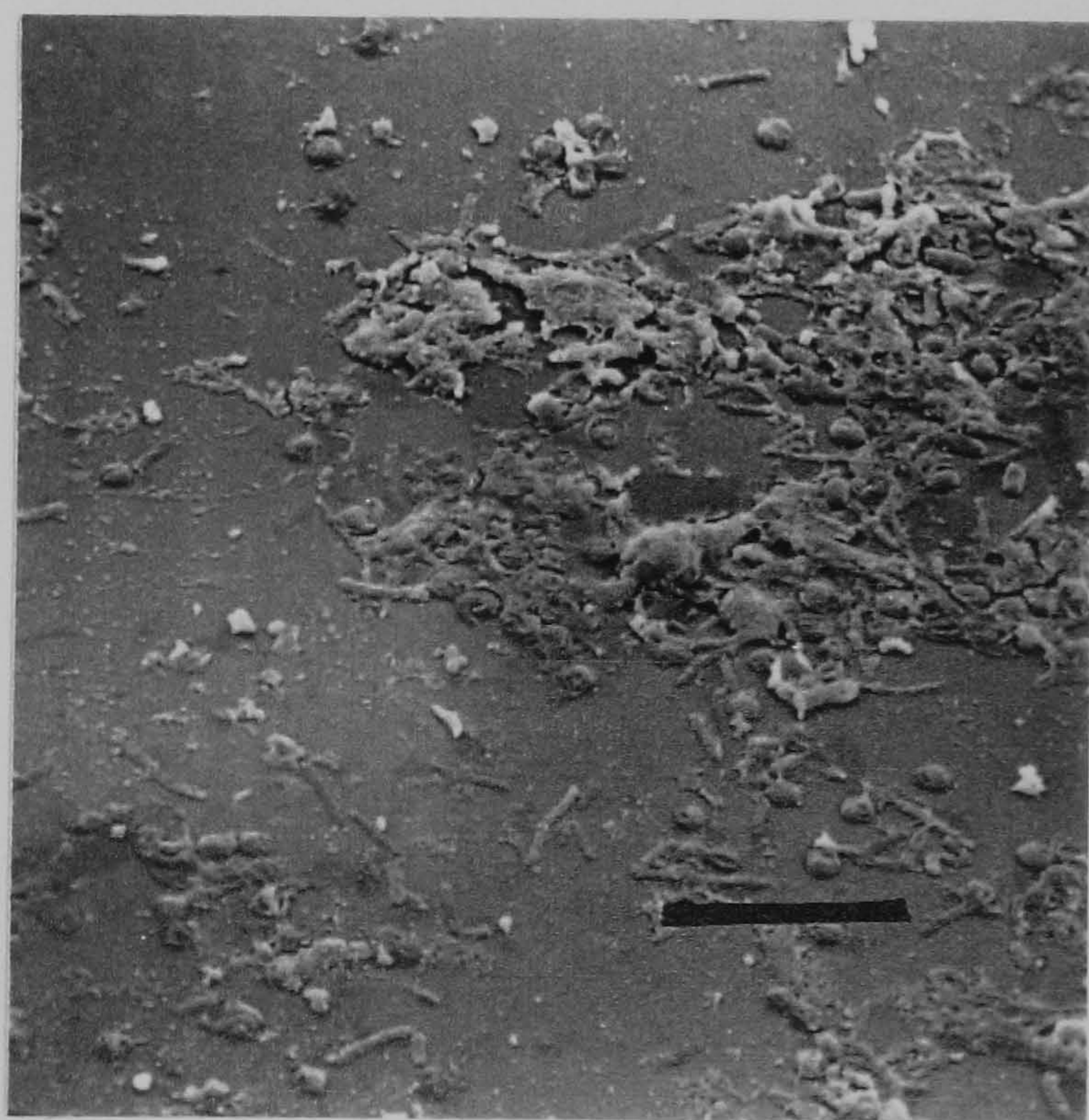
(A) 1 Day



(B) 7 Day

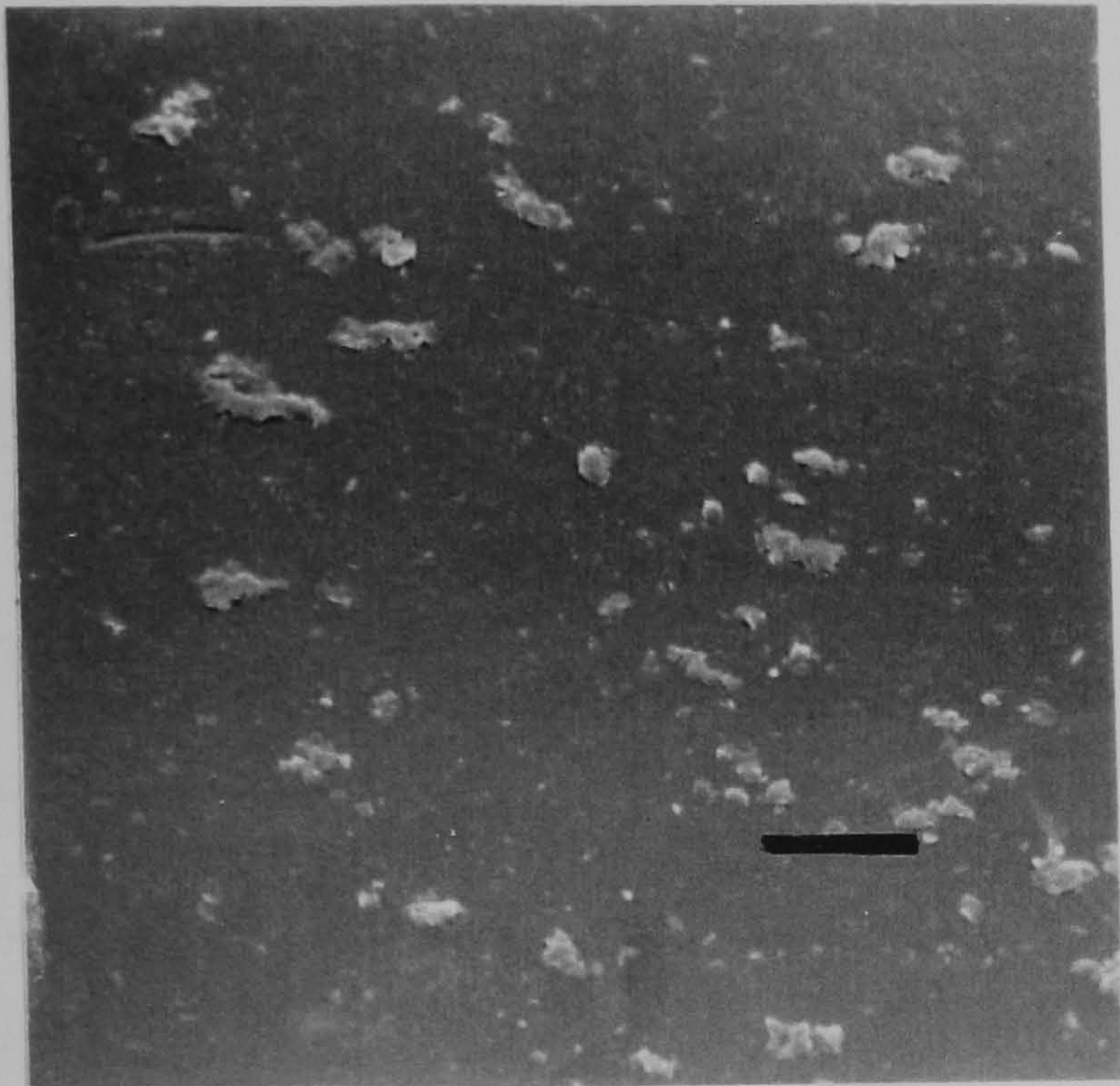


(C) 14 Day

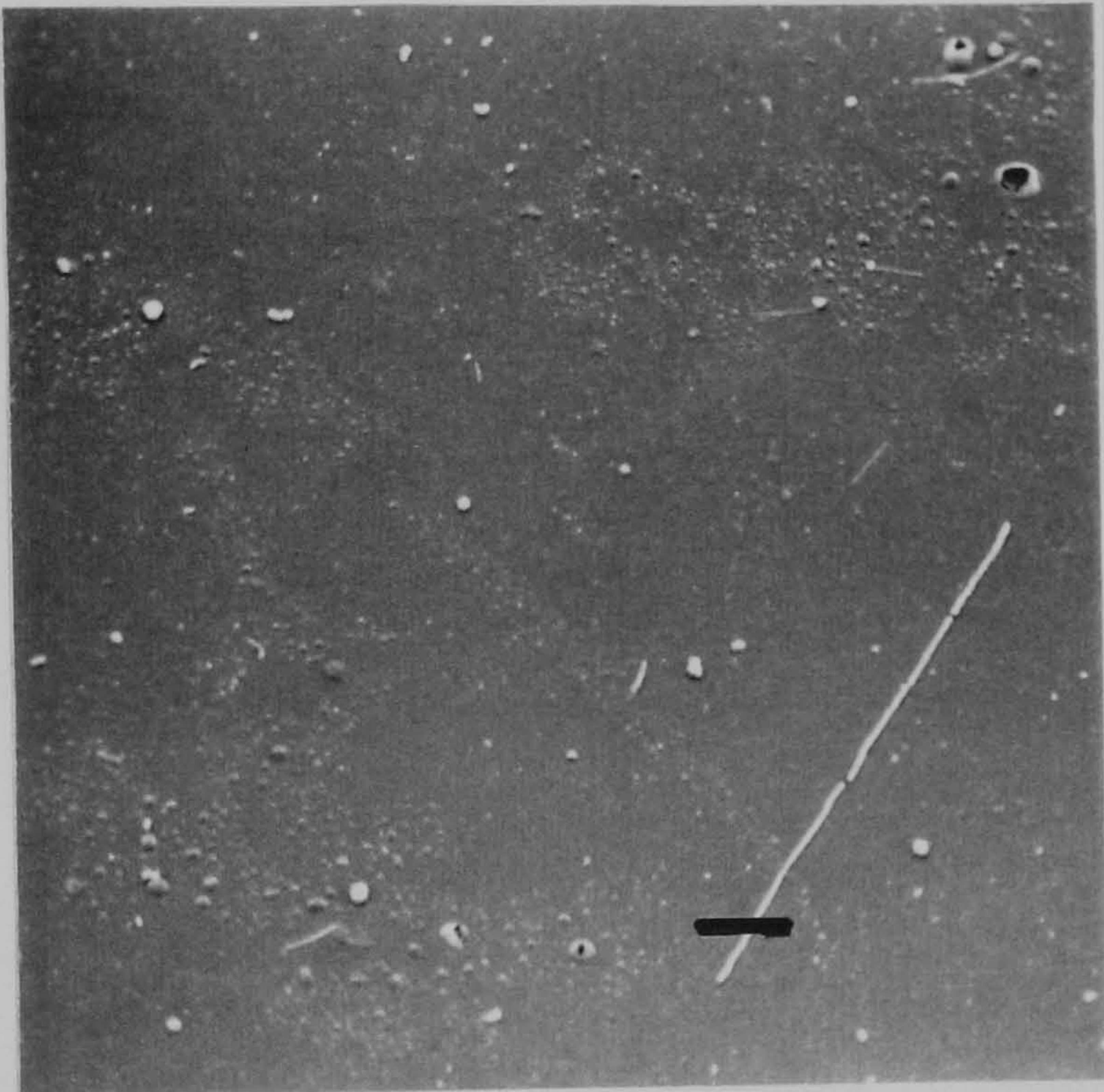


(D) 21 Day

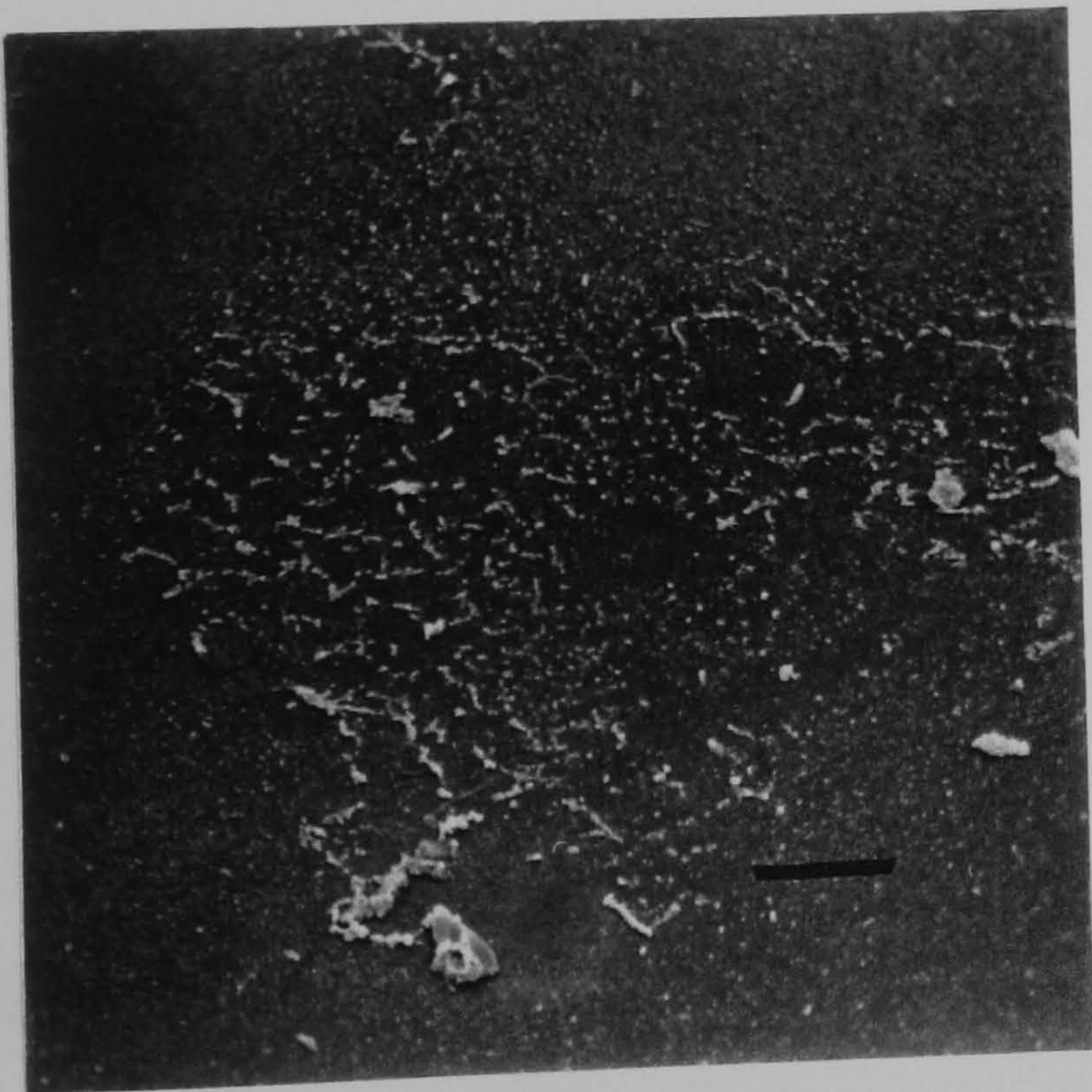
Figure 4.15 SEM of glass coupons exposed to temperatures of 60°C (Marker bar denotes 10 μm).



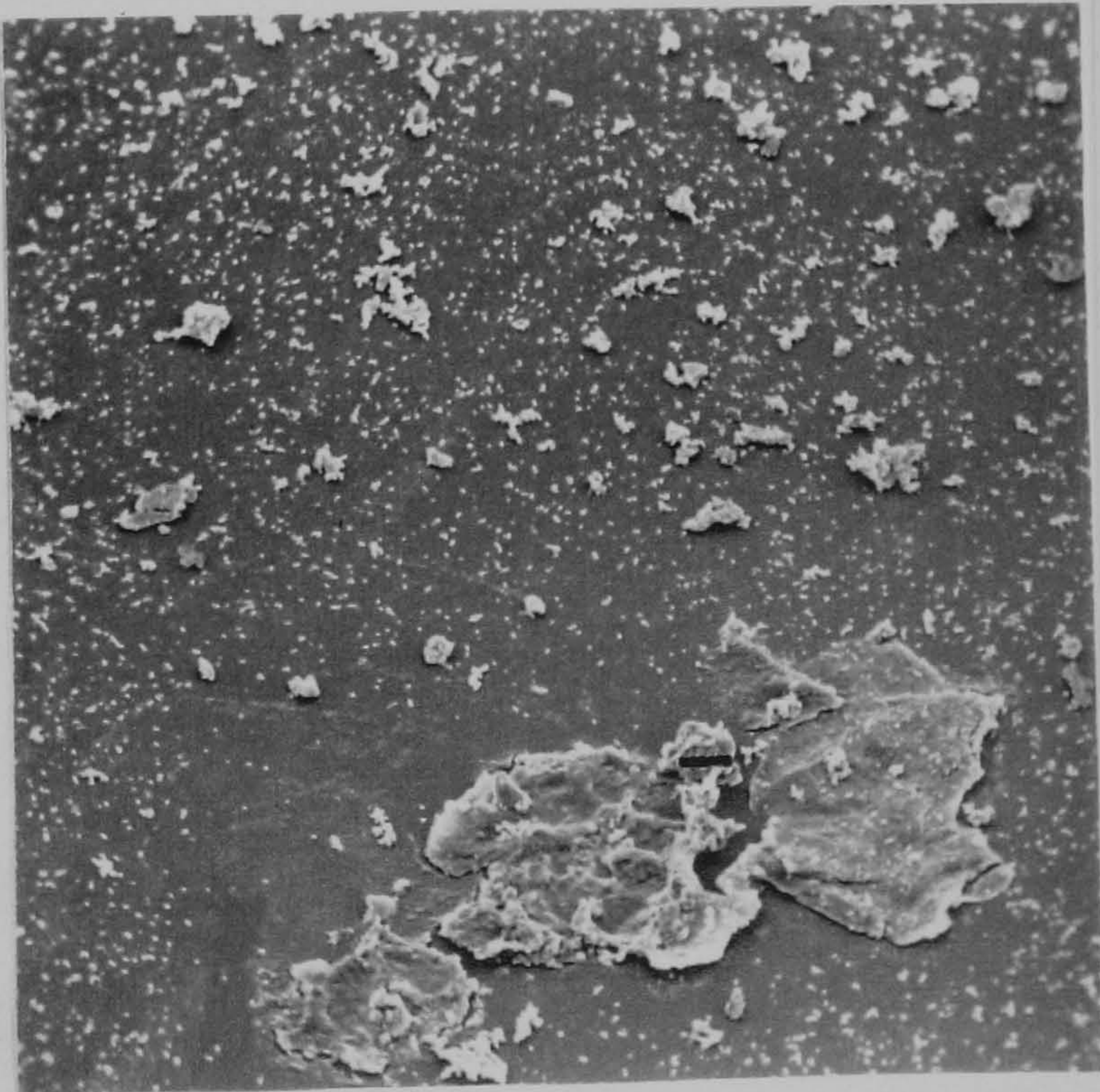
(A) 1 Day



(B) 7 Day



(C) 14 Day



(D) 21 Day

Table 4.12 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on glass at 40°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	59600	12800	4.77	2.54
7	68400	45300	4.8	2.9
14	30100	3500	4.47	3.7
21	50400	6900	4.7	3.26

Table 4.13 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on glass at 45°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	40800	25500	4.6	3.84
7	17100	14200	4.23	3.27
14	38815	2800	4.6	3.37
21	15540	24300	4.19	2.6

Table 4.14 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on glass at 50°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	1425	1425	3.45	1.69
7	70200	33000	4.84	3.07
14	22950	15000	4.36	2.6
21	32760	6384	4.51	1.3

Table 4.15 Counts extrapolated from the total number of bacteria observed on the electron micrographs of biofilm generated on glass at 55°C

Day	Total Count	Error(±)	Log	Viable Count (Log)
1	4400	2800	3.64	3.66
7	31080	9250	4.5	2.3
14	55000	70404	4.74	2.39
21	53460	16170	4.72	4.88

4.4 REPEAT OF BIOFILM DEVELOPMENT BETWEEN 40-60°C.

4.4.1 Repeat biofilm development of copper surfaces

After studying colonisation of glass and copper between 40°C and 60°C the temperature was raised up to 80°C in 5.0°C increments before returning to 40°C. The ability of the bacteria to colonise the surface of copper and glass coupons was then examined again to see if exposure to increased temperatures had altered the profile of bacterial colonisation of the surfaces between 40 - 60°C

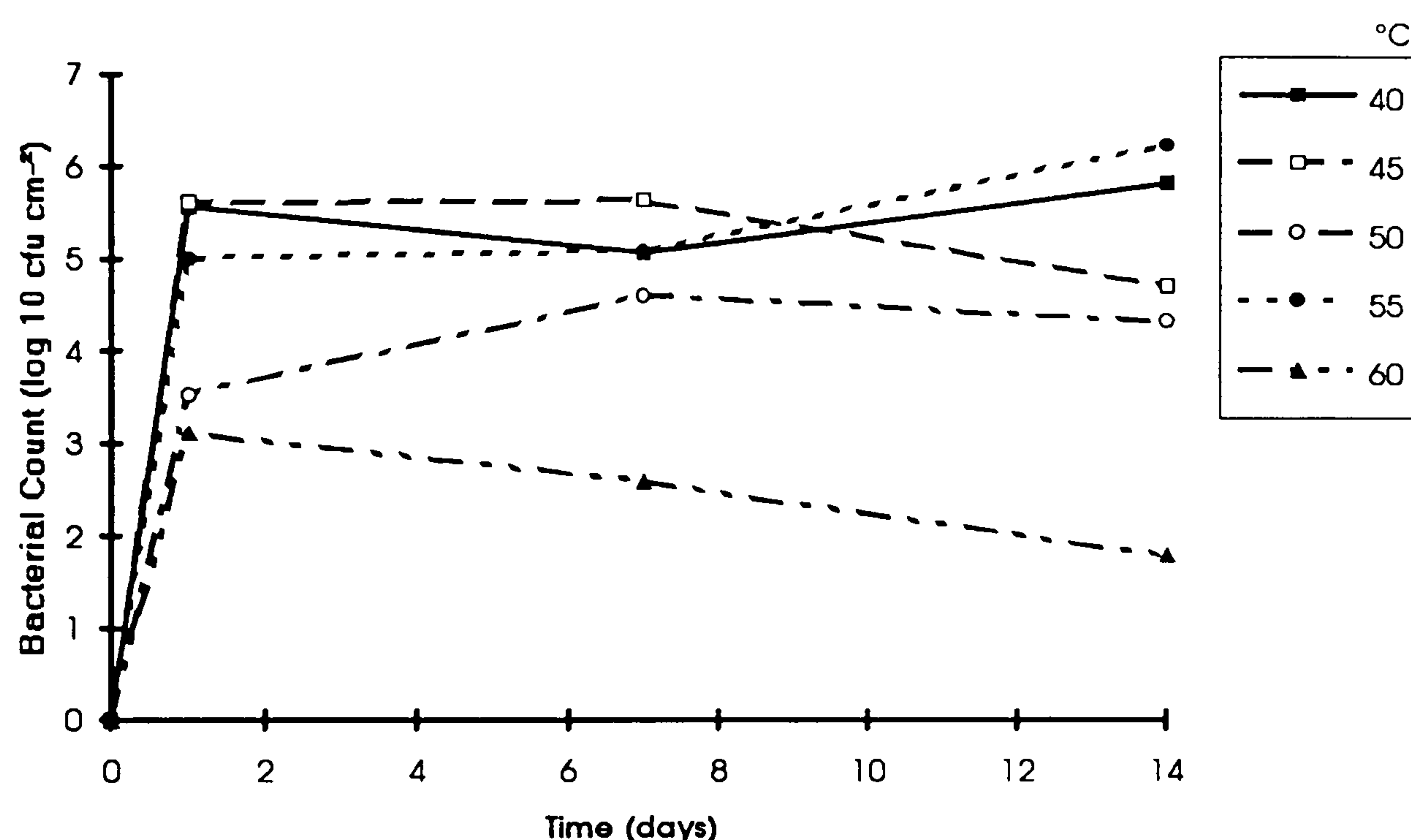


Figure 4.16 Colonisation of copper coupons in Glasgow water after temperature of culture had been increased to 80°C.

Rapid colonisation of the copper occurred at 3.5 - 5.8 Log 10 cfu cm⁻² (40-55°C) within 24 hours of the surfaces being immersed in the culture. Bacteria were recovered from the copper coupons between 40°C and 55°C. Bacterial numbers recovered at 40°C, 50°C and 55°C remained constant or increased (4.7 - 6.2 Log 10 cfu cm⁻²) until day 14. At 45°C the number of bacteria recovered at day 14

decreased by 0.9 Log₁₀ cfu cm⁻². Although bacteria were recovered from coupons which were exposed to the culture at 60°C, only 3.9 Log₁₀ cfu cm⁻² viable bacteria were detected at day 1 and this number decreased to 2.3 Log₁₀ cfu cm⁻² at day 14. There were no significant differences between the bacterial numbers recovered between 40 & 55°C and 45°C & 55°C ($p = >0.05$). However there were statistical difference between 40°C & 45°C, 40 & 50°C, 45 & 50°C, 50 & 55°C, 40 & 60°C, 45 & 60°C and 55 & 60°C where $p = <0.05$).

(i) Percentage profile of bacteria recovered from copper coupons at 40°C

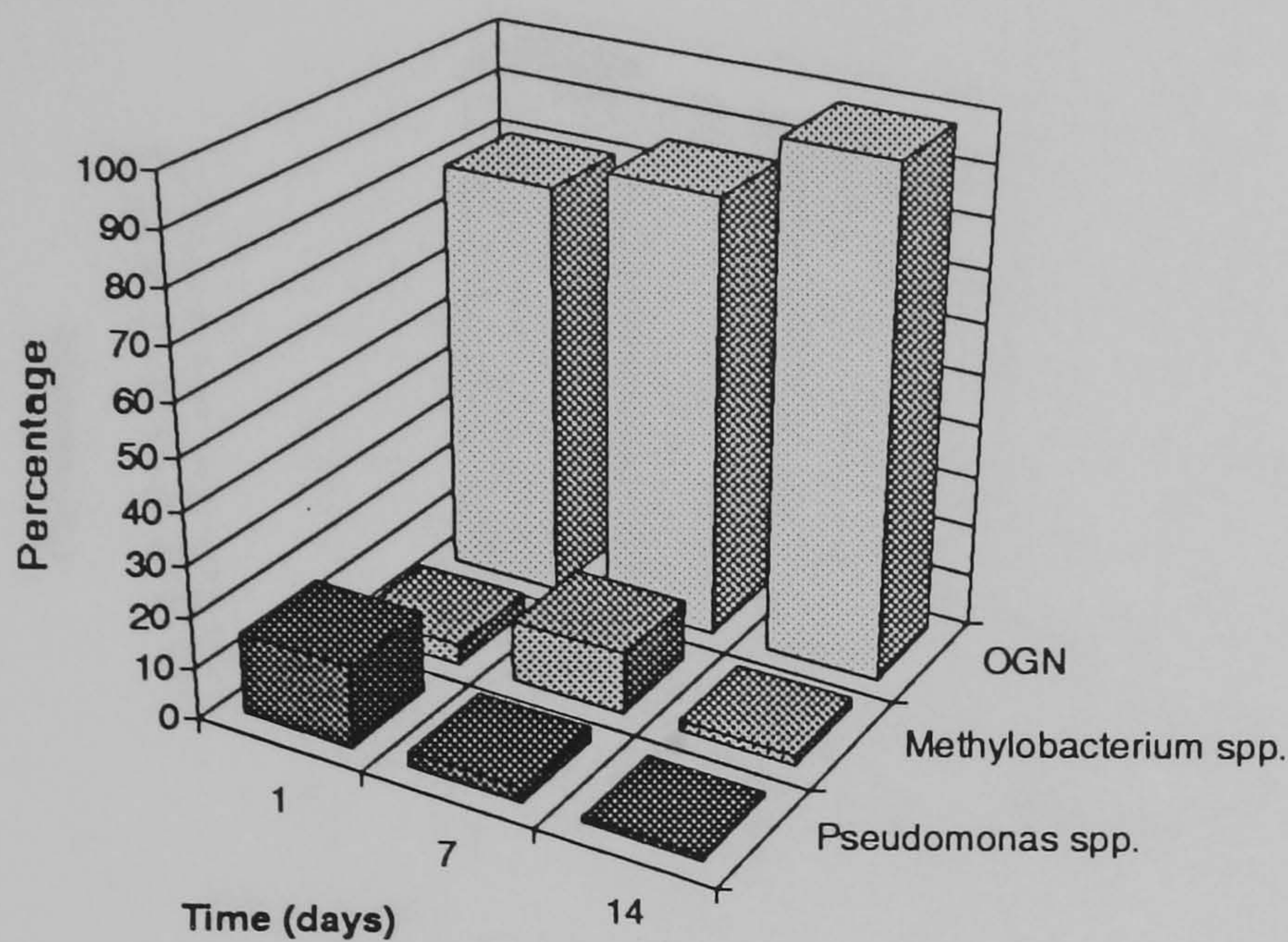


Figure 4.17 Percentage profile of bacteria on copper at 40°C in Glasgow water after 80°C temperature cycle.

Table 4.16 Percentage profile of microbial species on copper at 40°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	16	3	1
<i>Methylobacterium</i> spp.	4	12	2
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	80	85	97

Although the *Pseudomonas* spp. were present at 16 % at day 1 their proportions declined to 3 & 1 % at days 7 & 14 respectively. *Methylobacterium* spp. were present throughout all days with a maximum of 12 % at day 7, with the OGN bacteria predominating at 80, 85 and 97 % respectively.

(ii) Percentage profile of bacterial recovered from copper coupons at 45°C

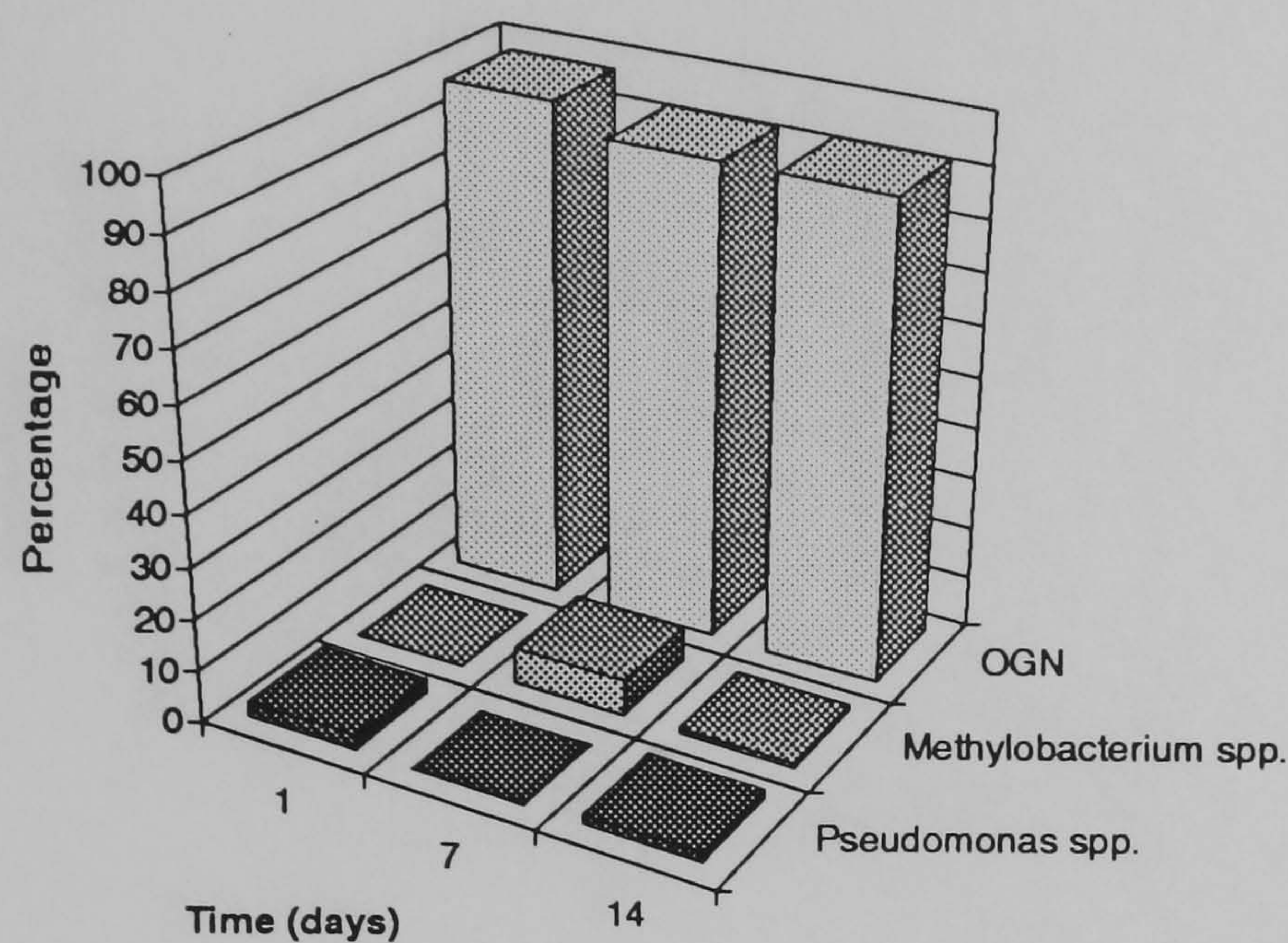


Figure 4.18 Percentage profile of bacteria on copper at 45°C in Glasgow water after 80°C temperature cycle.

Table 4.17 Percentage profile of microbial species on copper at 45°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	3	0	1
<i>Methylobacterium</i> spp.	0	7	2
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	97	93	97

OGN dominated from day 1 to day 14 at 97, 93 and 97 % respectively with *Pseudomonas* spp. and *Methylobacterium* spp. being present at less than 10%.

(iii) Percentage profile of bacteria recovered from copper coupons at 50°C

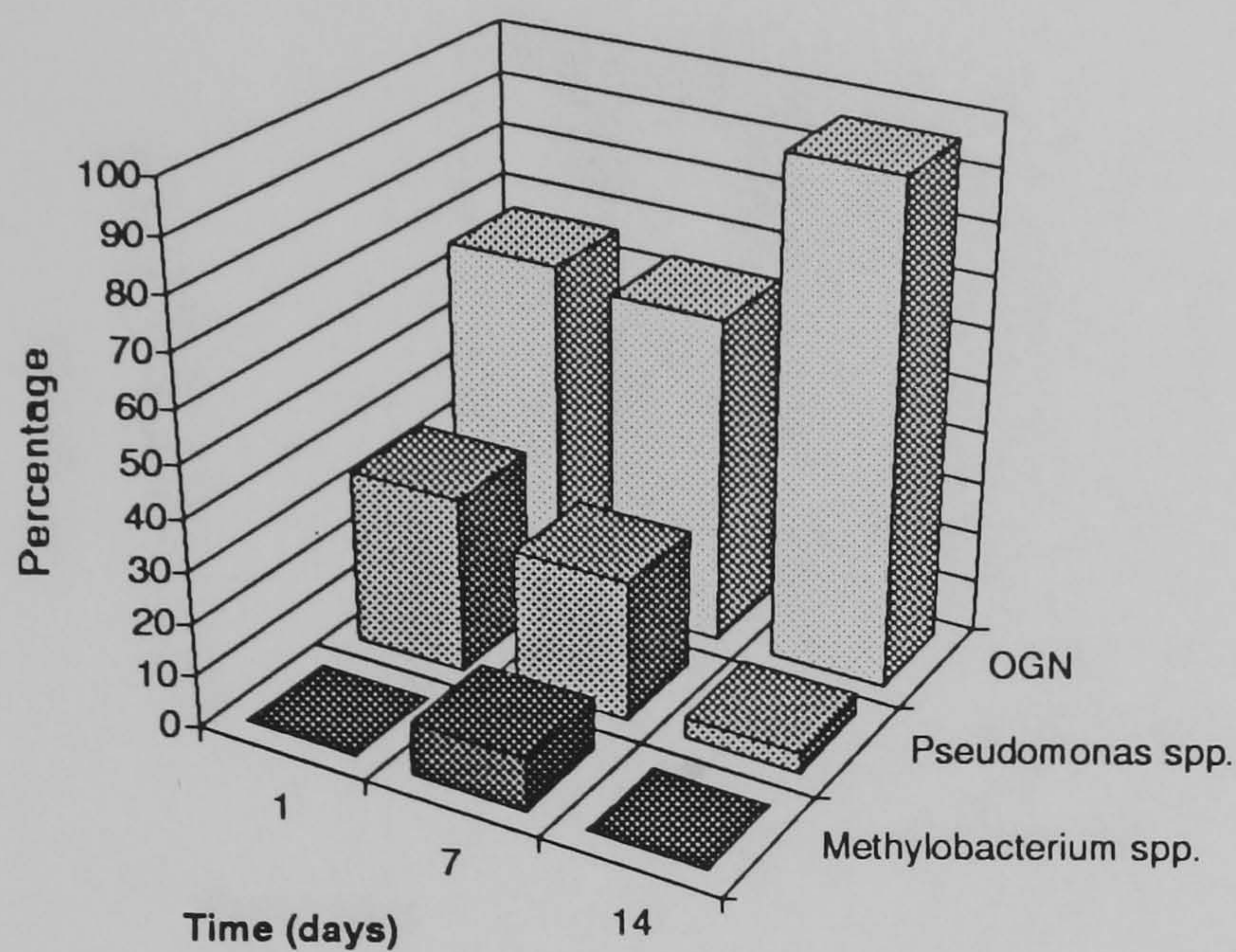


Figure 4.19 Percentage profile of bacteria on copper at 50°C in Glasgow water after 80°C temperature cycle.

Table 4.18 Percentage profile of microbial species on copper at 50°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	34	27	4
<i>Methylobacterium</i> spp.	0	10	0
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	66	63	96

Methylobacterium spp. were present at 10% at day 7 but were absent from the populations recovered at day 1 and 14. At day 1 the *Pseudomonas* spp. represented 34 % of the population but declined to 27 % and 4 % respectively. The OGN bacteria dominated from day 1 at 66%, day 7 at 63 % and 96 % at day 14.

(iv) Percentage profile of bacteria recovered from copper at 55°C

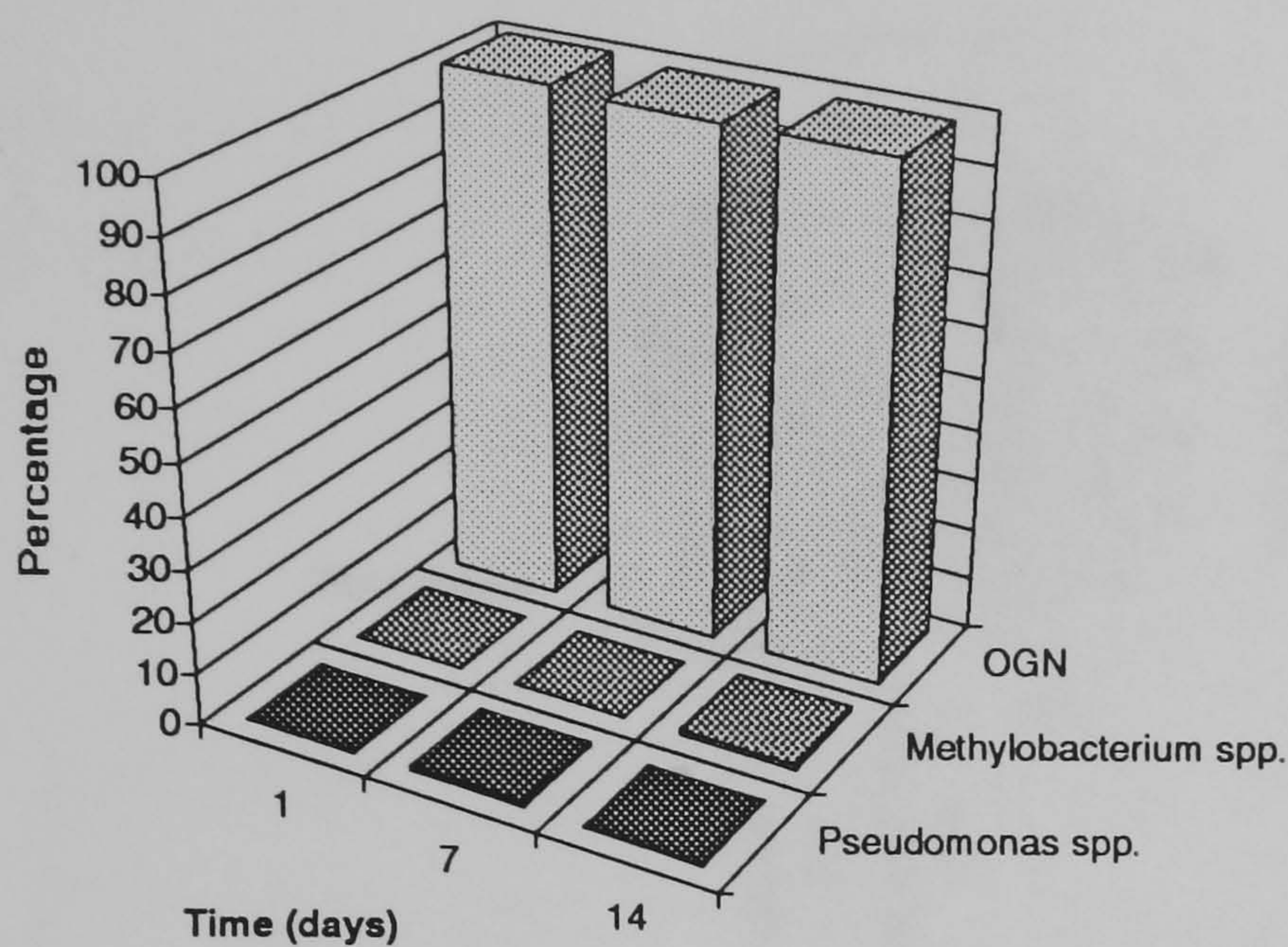


Figure 4.20 Percentage profile of bacteria on copper at 55°C in Glasgow water after 80°C temperature cycle.

Table 4.19 Percentage profile of microbial species on copper at 55°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	0	0	0
<i>Methylobacterium</i> spp.	0	0	1
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	100	100	99

The OGN bacteria dominated from day 1 to day 14 with only 1 % *Methylobacterium* spp. being present at day 14.

(v) Percentage profile of bacteria recovered from copper coupons at 60°C

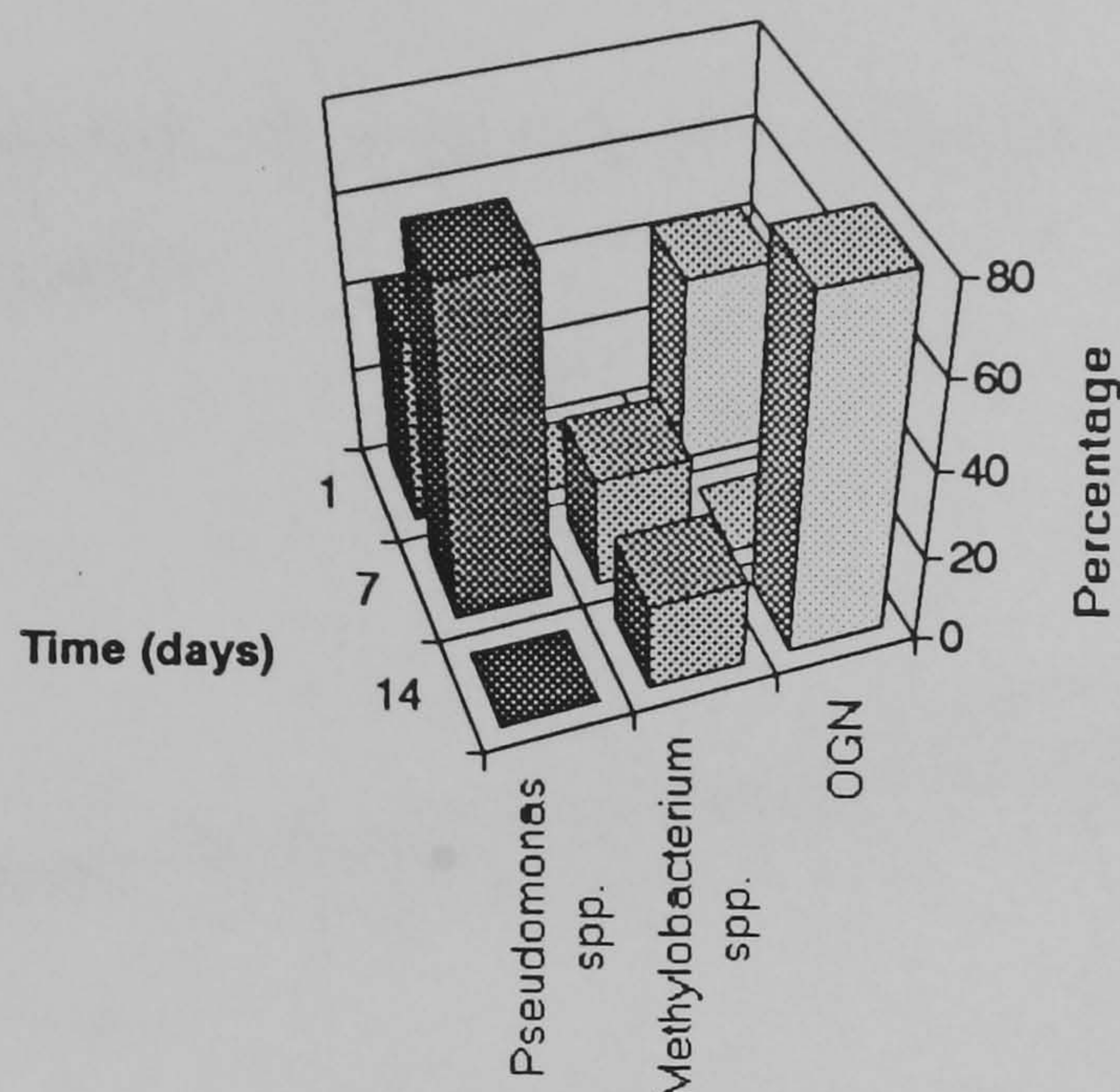


Figure 4.21 Percentage profile of bacteria on copper at 60°C in Glasgow water after 80°C temperature cycle.

Table 4.20 Percentage profile of microbial species on copper at 60°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	46	75	0
<i>Methylobacterium</i> spp.	0	25	20
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	54	0	80

Pseudomonas spp. and OGN bacteria were recovered at approximately 50 % on day 1 with the *Pseudomonas* spp. dominating at day 7 but were not detectable at day 14. At day 7 the OGN were not detected but succeeded at day 21 at 80 % of the population. The *Methylobacterium* spp. were not detected until day 7 at 25 % and remained present at 20 % of the total population at day 14.

4.4.2 Repeat biofilm development of glass surfaces

Recovery of viable bacteria from glass coupons immersed into the vessel after the culture was exposed to 80°C.

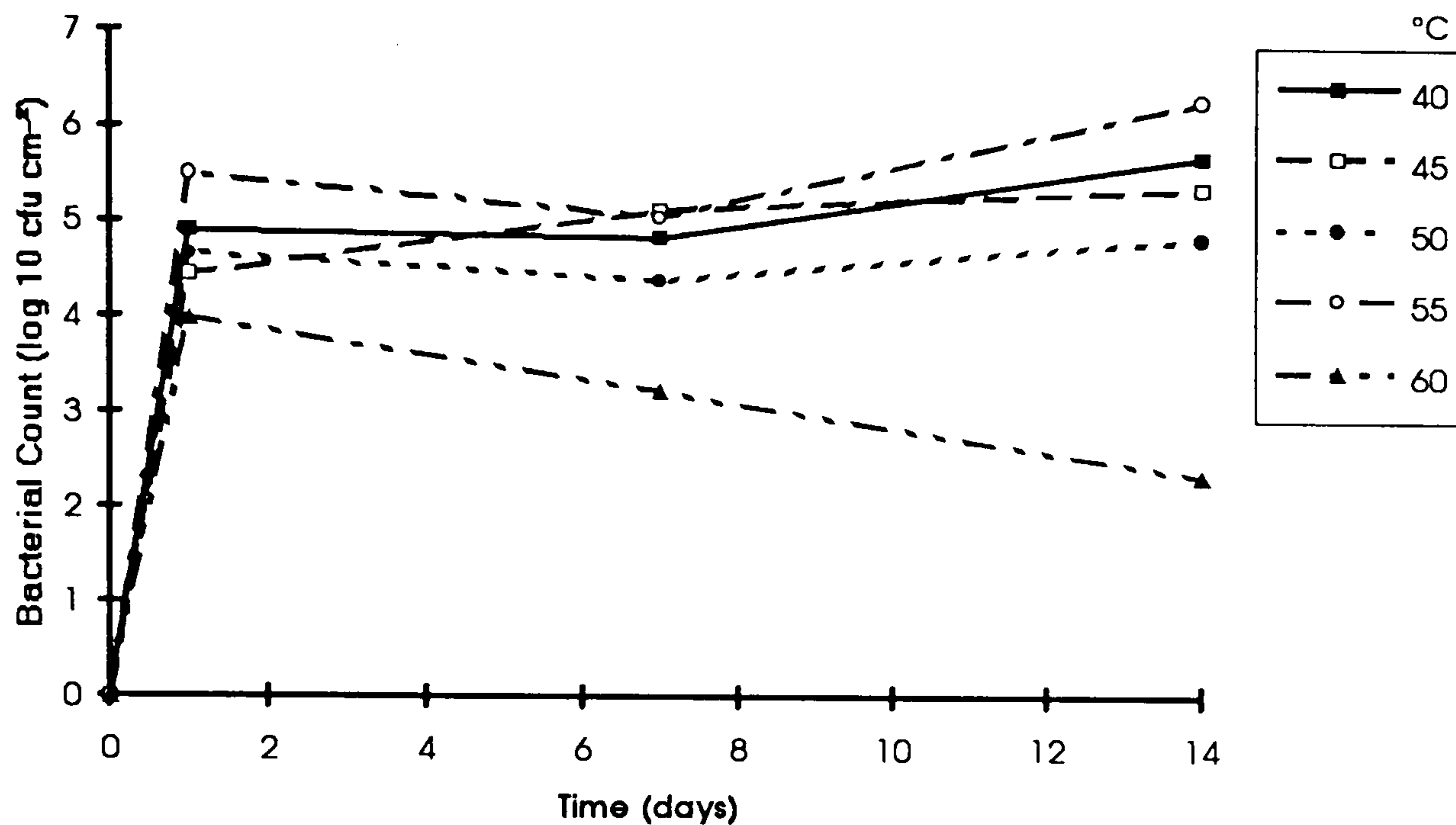


Figure 4.22 Colonisation of glass in the presence of copper after 80°C temperature cycle.

Recovery of bacteria from the glass control surfaces ranged from 3.9 - 5.5 Log₁₀ cfu cm⁻² at all temperatures between 40-60°C, indicating rapid colonisation. At day 7 the number of bacteria recovered at 60°C had decreased to 3.6 Log₁₀ cfu cm⁻² but between 40 - 55°C the number remained relatively constant in ranging from 4.7 - 5.8 Log₁₀ cfu cm⁻². The number of bacteria recovered at 60°C decreased again to 2.8 Log₁₀ cfu cm⁻² at day 14. However the recovery of bacteria at day 14 ranged from 4.9 - 6.7 Log₁₀ cfu cm⁻² exhibiting an increase at all temperatures between 40-60°C. There was no significant difference between the bacterial numbers retrieved from coupons at 40 & 45°C or 45 & 50°C ($p = >0.05$). In comparison a statistical difference was apparent between 40 & 50°C, 40 & 55°C, 50 & 55°C, 40 & 60°C, 45 & 60°C, 50 & 60°C and 55 & 60°C where $p = <0.05$).

(i) Percentage profile of bacteria recovered from glass at 40°C

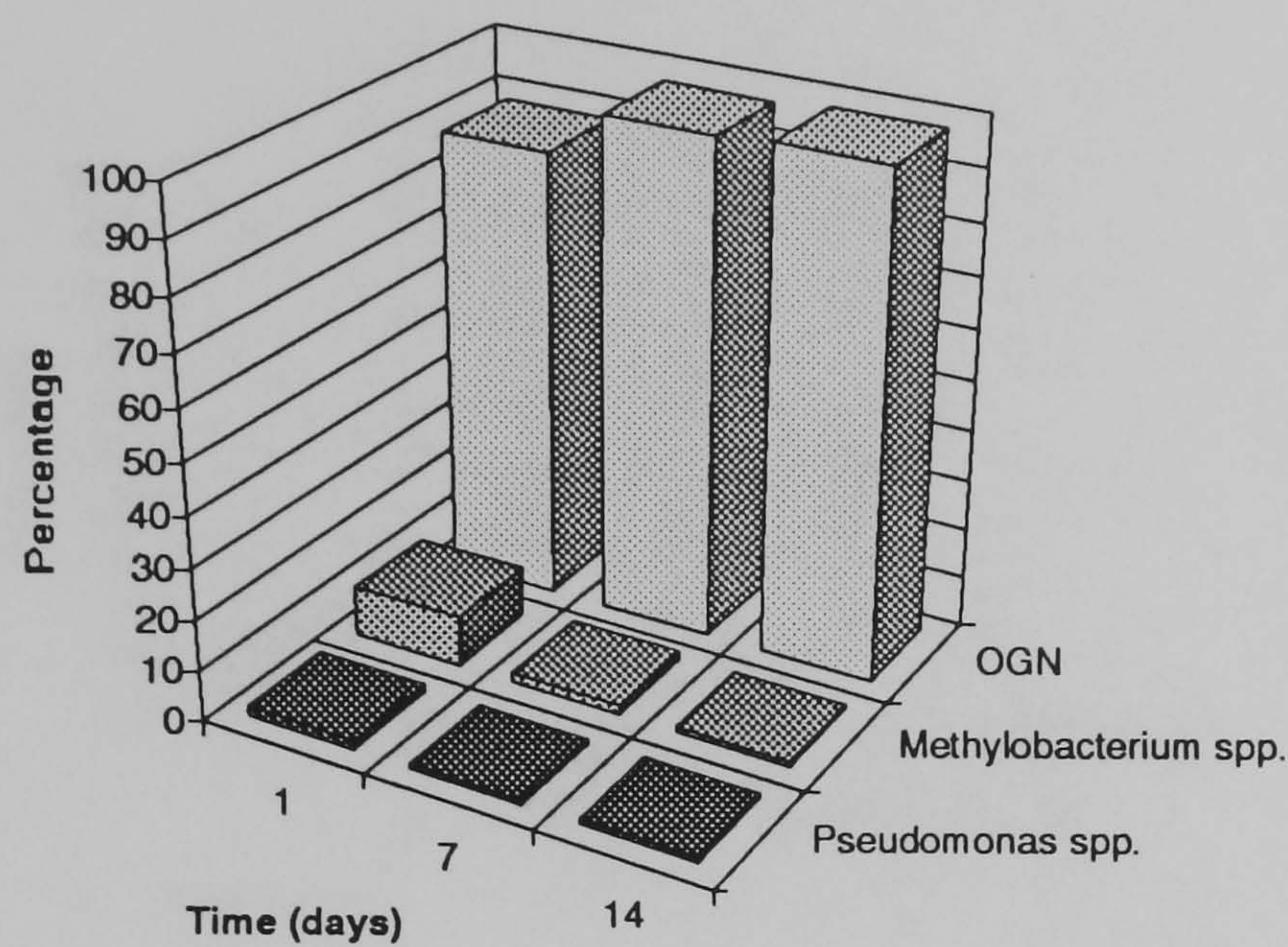


Figure 4.23 Percentage profile of bacteria on glass at 40°C in Glasgow water after 80°C temperature cycle.

Table 4.21 Percentage profile of microbial species on glass at 40°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	2	1	1
<i>Methylobacterium</i> spp.	10	2	1
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	88	97	98

At 40°C the OGN bacteria dominated at > 97 % of the consortia. *Methylobacterium* spp. were present at 10 % of the population but decreased to 2 % then 1 % by day 7 and 14 respectively. Recovery of *Pseudomonas* spp. was at less than 2 % of the total population.

(ii) Percentage profile of bacteria recovered from glass coupons at 45°C

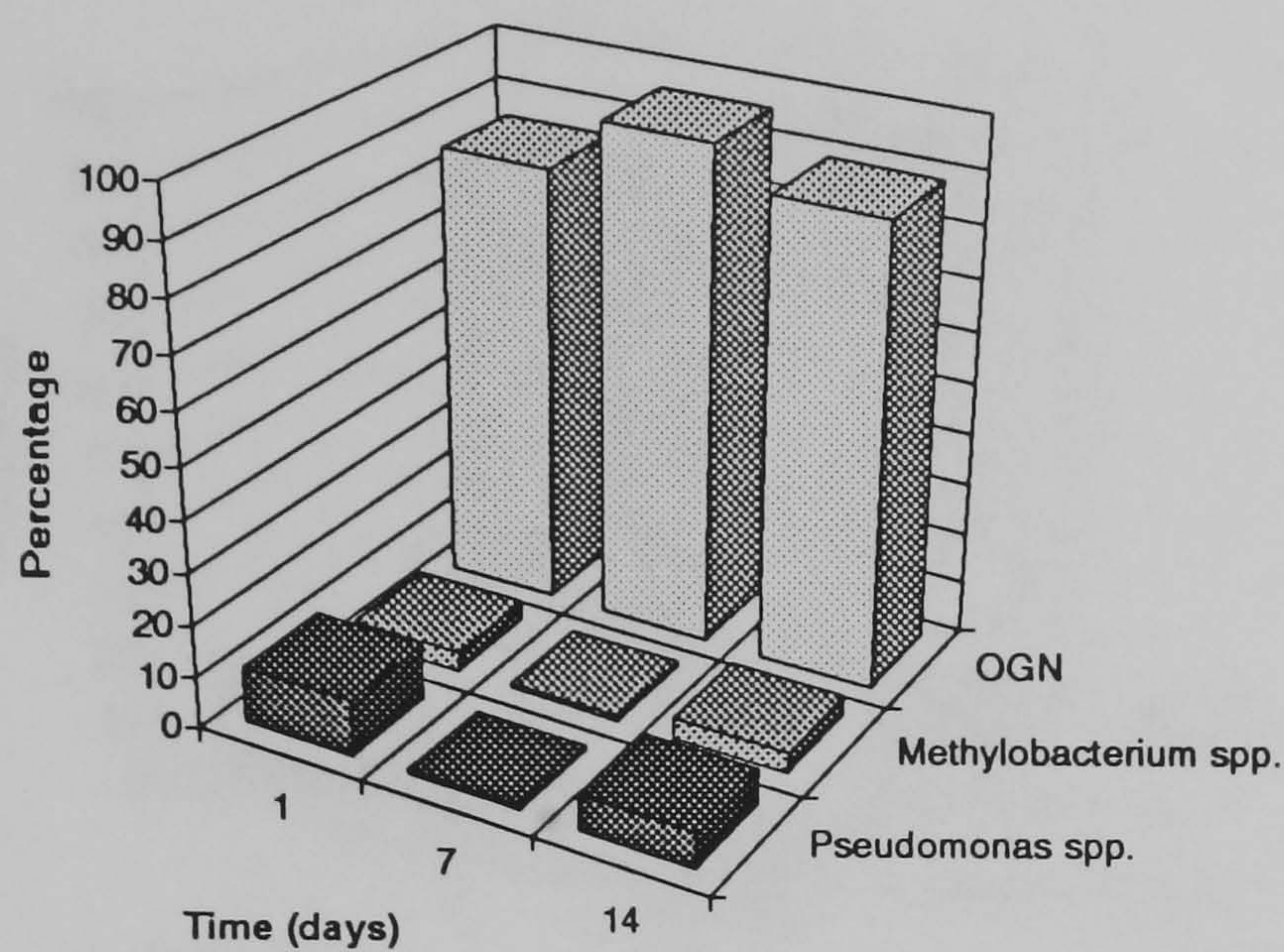


Figure 4.24 Percentage profile of bacteria on glass at 45°C in Glasgow water after 80°C temperature cycle.

Table 4.22 Percentage profile of microbial species on glass at 45°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	11	1	7
<i>Methylobacterium</i> spp.	4	1	4
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	85	98	89

From day 1 to day 14 the OGN bacteria dominated at 85 %, 96 % and 89 %. *Methylobacterium* spp. and *Pseudomonas* spp. were recovered at < 11 % of the population.

(iii) Percentage profile of bacteria recovered from glass at 50°C

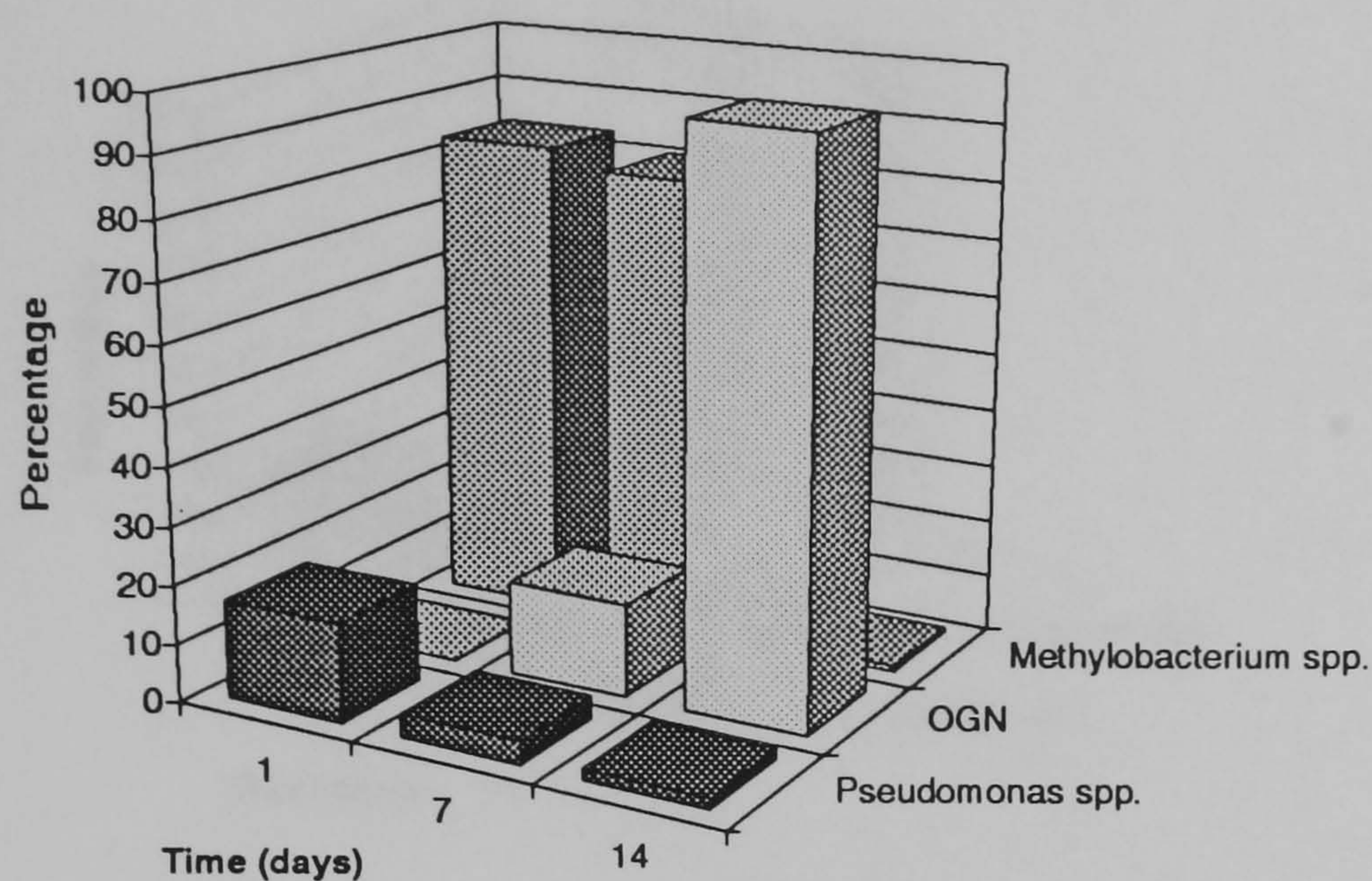


Figure 4.25 Percentage profile of bacteria on glass at 50°C in Glasgow water after 80°C temperature cycle.

Table 4.23 Percentage profile of microbial species on glass at 50°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	17	4	2
<i>Methylobacterium</i> spp.	0	79	1
<i>Aspergillus fumigatus</i>	0	1	0
Gram negative bacteria	83	16	97

Although the *Methylobacterium* spp. dominated at day 1, 83 %, they were succeeded by the OGN bacteria at day 7 but dominated again at day 14 at 97 %. *Pseudomonas* spp. were recovered at 17 % of the population at day 1 but decreased to 4 & 2 % at day 7 & 14.

(iv) Percentage profile of bacteria recovered from 55°C

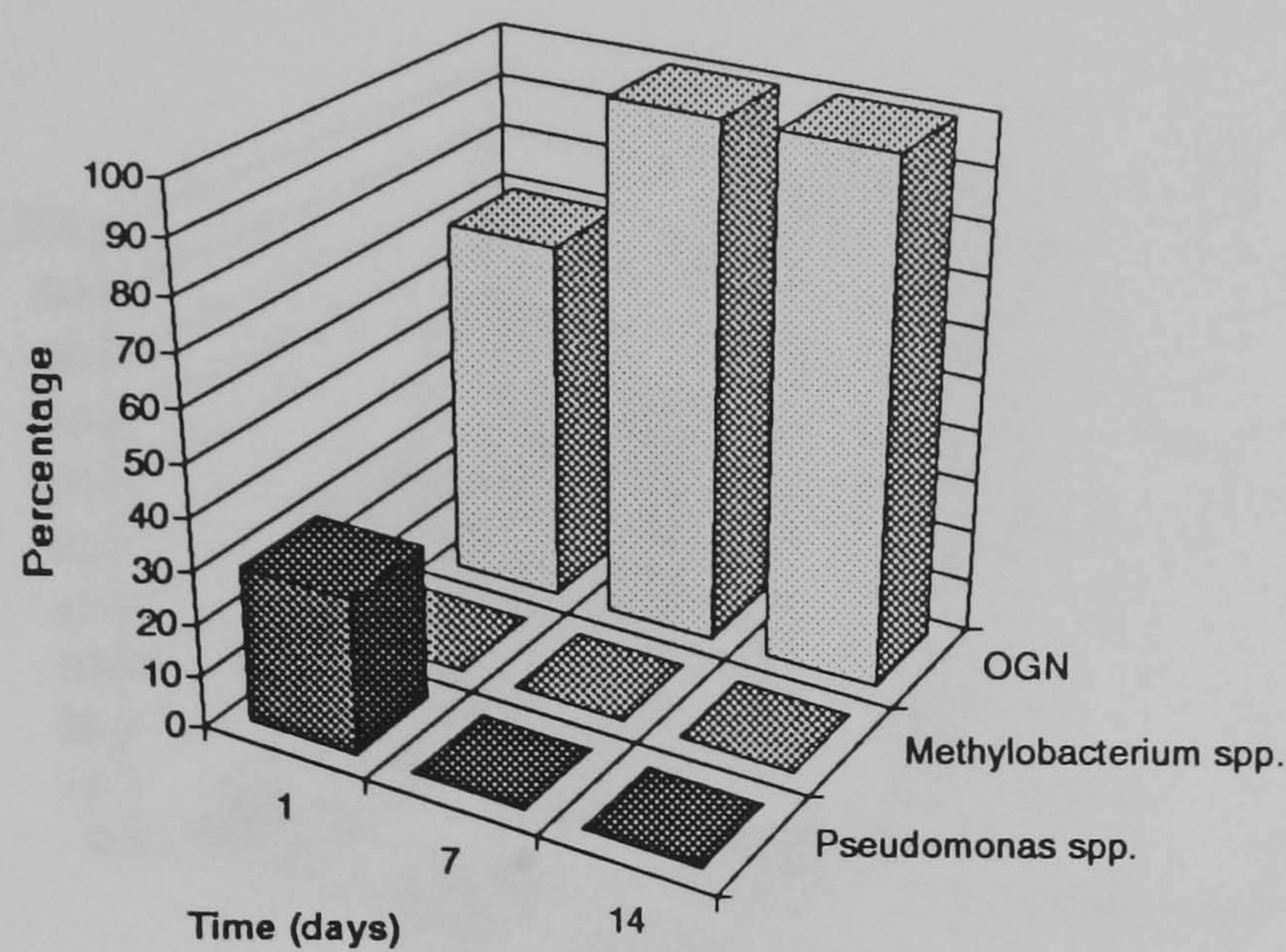


Figure 4.26 Percentage profile of bacteria on glass at 55°C in Glasgow water after 80°C temperature cycle.

Table 4.24 Percentage profile of microbial species on glass at 55°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	30	0	0
<i>Methylobacterium</i> spp.	0	0	0
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	70	100	100

The OGN bacteria dominated at 70 and 100%. *Pseudomonas* spp. were only recovered at day 1 at 30 %.

(v) Percentage profile of bacteria recovered from glass tile immersed in the culture at 60°C

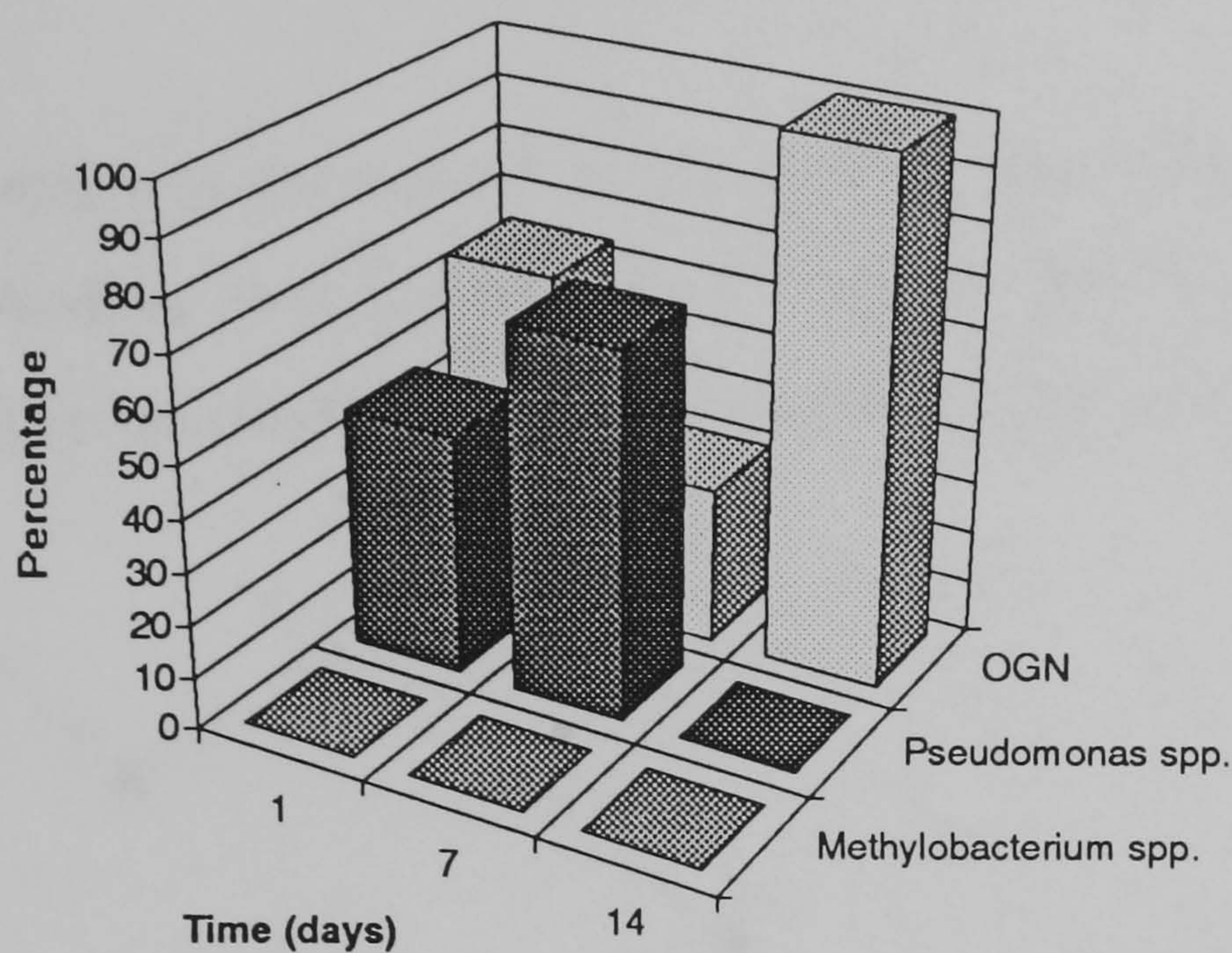


Figure 4.27 Percentage profile of bacteria on glass at 60°C in Glasgow water after 80°C temperature cycle.

Table 4.25 Percentage profile of microbial species on glass at 60°C.

Species / Days	1	7	14
<i>Pseudomonas</i> spp.	46	70	0
<i>Methylobacterium</i> spp.	0	0	0
<i>Aspergillus fumigatus</i>	0	0	0
Gram negative bacteria	64	30	100

At day 1 the OGN bacteria dominated at 64 % compared to 46 % of *Pseudomonas* spp. By day 7 the OGN bacteria were present at 30 % and had been succeeded by the *Pseudomonas* spp. at 70 %. However the OGN dominated at day 14 at 100 %. No *Methylobacterium* spp. were recovered.

4.5 PLANKTONIC POPULATIONS IN THE CONTINUOUS CULTURE VESSELS

As bacteria present in the aqueous environment are those primarily responsible for establishing biofilms over the temperature range of 40-55°C, bacteria within the planktonic phase were examined for their response to varying temperatures.

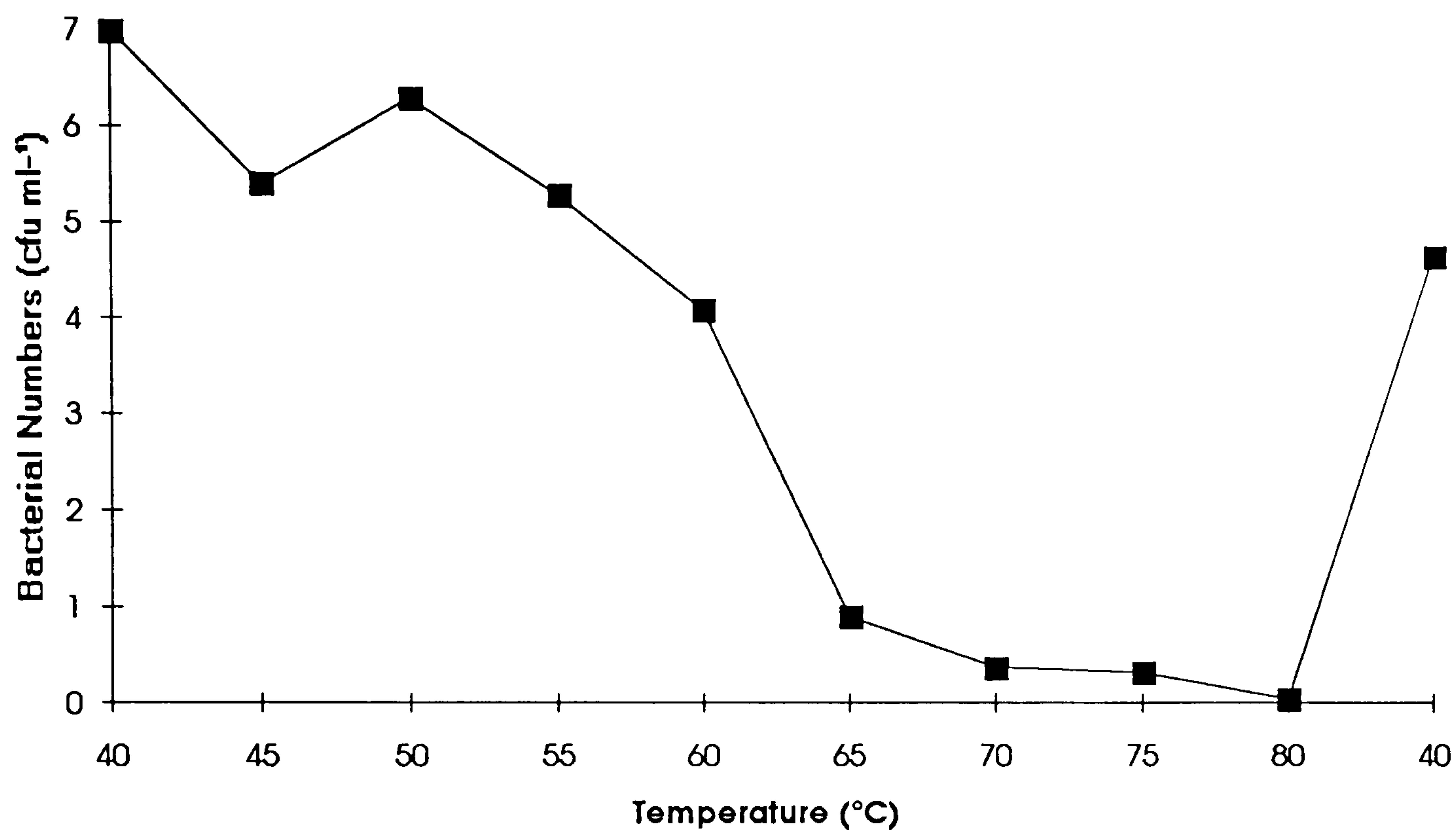


Figure 4.28 Effect of temperature changes on bacterial numbers in the planktonic phase (each value represents the mean of at least three separate measurements).

From a mean value of 7.0×10^5 cfu ml⁻¹ at 40°C there was a drop in the number of bacteria to 5.4×10^5 cfu ml⁻¹ as the temperature was increased to 45°C. This was followed by an increase to 6.3×10^5 cfu ml⁻¹ at 50°C. When the temperature was changed to 55°C the number of bacteria recovered was 5.3×10^5 cfu ml⁻¹ with 4.1×10^5 cfu ml⁻¹ at 60°C. However increasing the temperature to 65, 70, 75 and 80°C resulted in a decrease in the number of viable bacteria recovered from the aqueous phase to 9.1×10^4 , 3.8×10^4 , 3.2×10^4 and 4.6×10^3 cfu ml⁻¹ respectively. Following a temperature change to 40°C, 4.7×10^5 cfu ml⁻¹ were present.

Table 4.26 Number of planktonic bacteria in vessels 1 and 2.

Vessel 1(cfu/ml) 30°C	log ₁₀ (cfu/ml)	Vessel 2 Temp (°C)	Vessel 2 (cfu/ml)	Log 10 (cfu/ml)
6.0 x 10 ⁵	5.78	40	7.0 x 10 ⁵	5.84
6.4 x 10 ⁵	5.80	45	5.4 x 10 ⁵	5.73
5.2 x 10 ⁵	5.72	50	6.3 x 10 ⁵	5.79
4.4 x 10 ⁵	5.64	55	5.3 x 10 ⁵	5.72
4.0 x 10 ⁵	5.60	60	4.5 x 10 ⁵	5.65
3.6 x 10 ⁵	5.56	65	9.1 x 10 ⁴	4.7
4.6 x 10 ⁵	5.66	70	3.8 x 10 ⁴	4.58
1.5 x 10 ⁵ *	5.17	75	3.3 x 10 ⁴	4.50
no count	no count	80	4.6 x 10 ³	3.66
2.4x 10 ⁵ *	5.34	40	4.7 10 ⁵	5.67

* denotes less than three counts per average per sample

The number of bacteria present in vessel one, where the temperature was maintained at 30°C ranged from 1.5 x 10⁵ cfu ml⁻¹ to 6.4 x 10⁵ cfu ml⁻¹, an average of 4.9 x 10⁵ cfu ml⁻¹ (5.6 log 10) over the first seven months sampling. In the second vessel where the temperature was increasing from 40°C in 5°C increments the number of bacteria present in the planktonic phase varied from 7.0 x 10⁵ cfu ml⁻¹ at 40°C to 4.6 x 10³ cfu ml⁻¹ at 80°C. When the temperature of the vessel was returned to 40°C the bacterial numbers recovered were found to be greater than 4.0 x 10⁵ cfu ml⁻¹.

(i) Percentage profile of planktonic phase during temperature fluctuations.

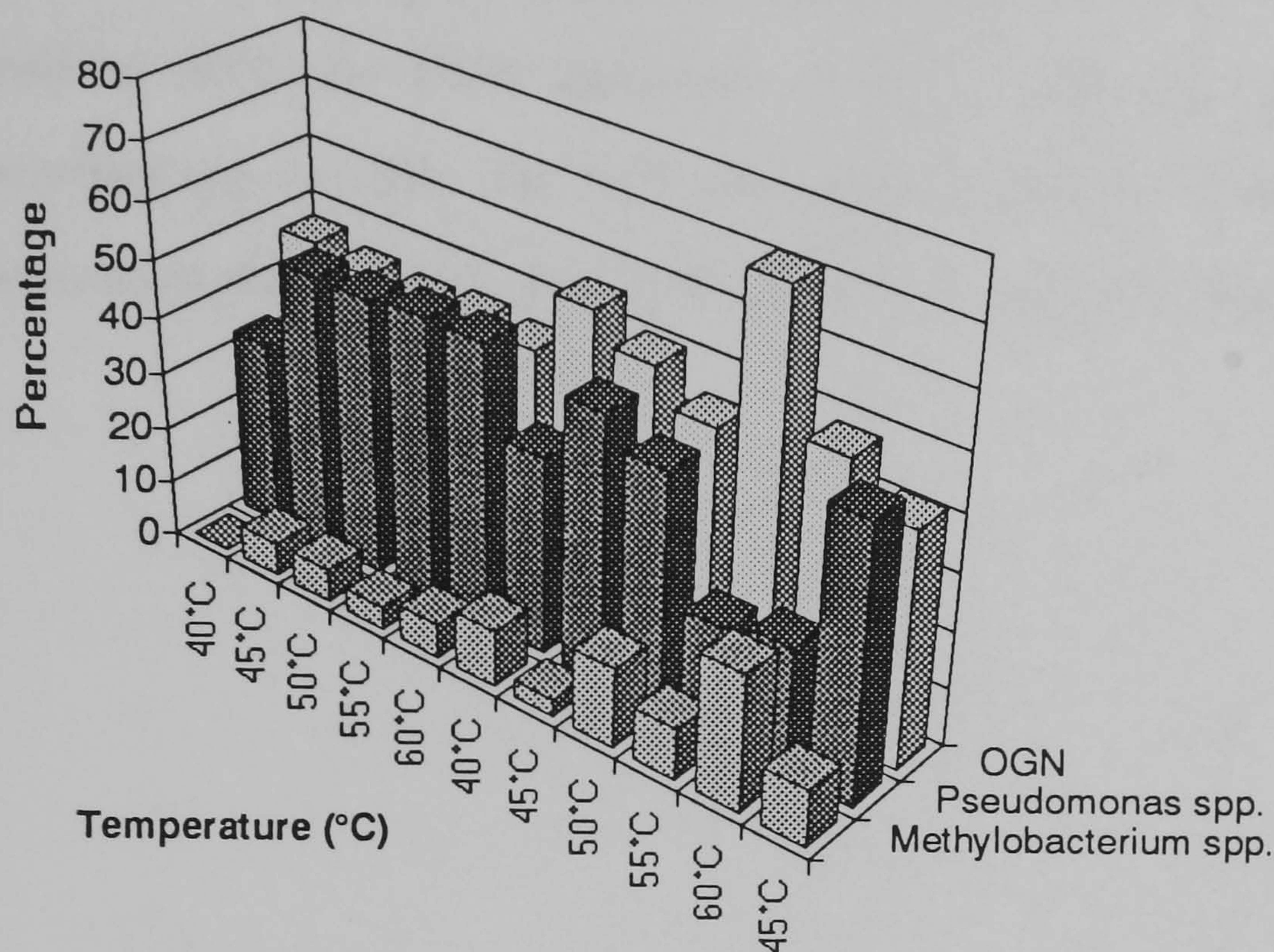


Figure 4.29 Percentage profile of bacteria in the planktonic phase during temperature cycling (each value represents the mean of at least three separate measurements).

Table. 27 Percentage profiles of bacterial species in the planktonic species during temperature cycling.

Species / Temperature	40°C	45°C	50°C	55°C	60°C	40°C	45°C	50°C	55°C	60°C	45°C
<i>Methylobacterium</i> spp.	0	6	6	4	6	10	3	14	9	25	10
<i>Pseudomonas</i> spp.	41	49	49	50	50	35	47	42	20	23	48
Gram negative	59	45	45	46	44	55	50	44	71	50	42

In the water phase the populations of bacteria present were the OGN, *Pseudomonas* spp. and *Methylobacterium* spp. During the temperature increments, the OGN represented 48.5 % of the population with *Pseudomonas* spp. at 40.5 % of the population on average. *Methylobacterium* spp. were present on average at 8.3 % of

the total population. During the first temperature gradient of 40-60°C, *Pseudomonas* spp. and OGN were in equal percentages of the population but when the temperature was returned to 40°C the OGN dominated at 55°C, with an increase in the *Methylobacterium* spp. to 10%. The OGN dominated again at 55°C and 60°C in the second temperature gradient with the *Methylobacterium* spp. increasing to 25 % at 60°C.

4.6 BIOFILM CONTROL

4.6.1 Control of fouling by Pasteurisation

From monitoring the hot water circuit in the hospital where the pepper pot corrosion was occurring it was established that the water temperature was maintained between 35-55°C. Using the laboratory model it was demonstrated that bacteria would form a biofilm between 40-55°C but not at 60°C. After repeating the experiment of biofouling between 40-60°C bacteria were recovered from surfaces immersed in the culture at 60°C, but the numbers were still reduced compared to the bacterial numbers at the lower temperatures. Therefore 60°C was chosen to study the effect of heat treatment by pasteurising established biofilms. Initially the biofilms were established in the chemostat at 45°C for 14 days before exposure to 60°C. To investigate if temperature fluctuations would alter the effect of pasteurisation, biofilms were matured between the temperatures of 40-60°C for 14 days before being pasteurised

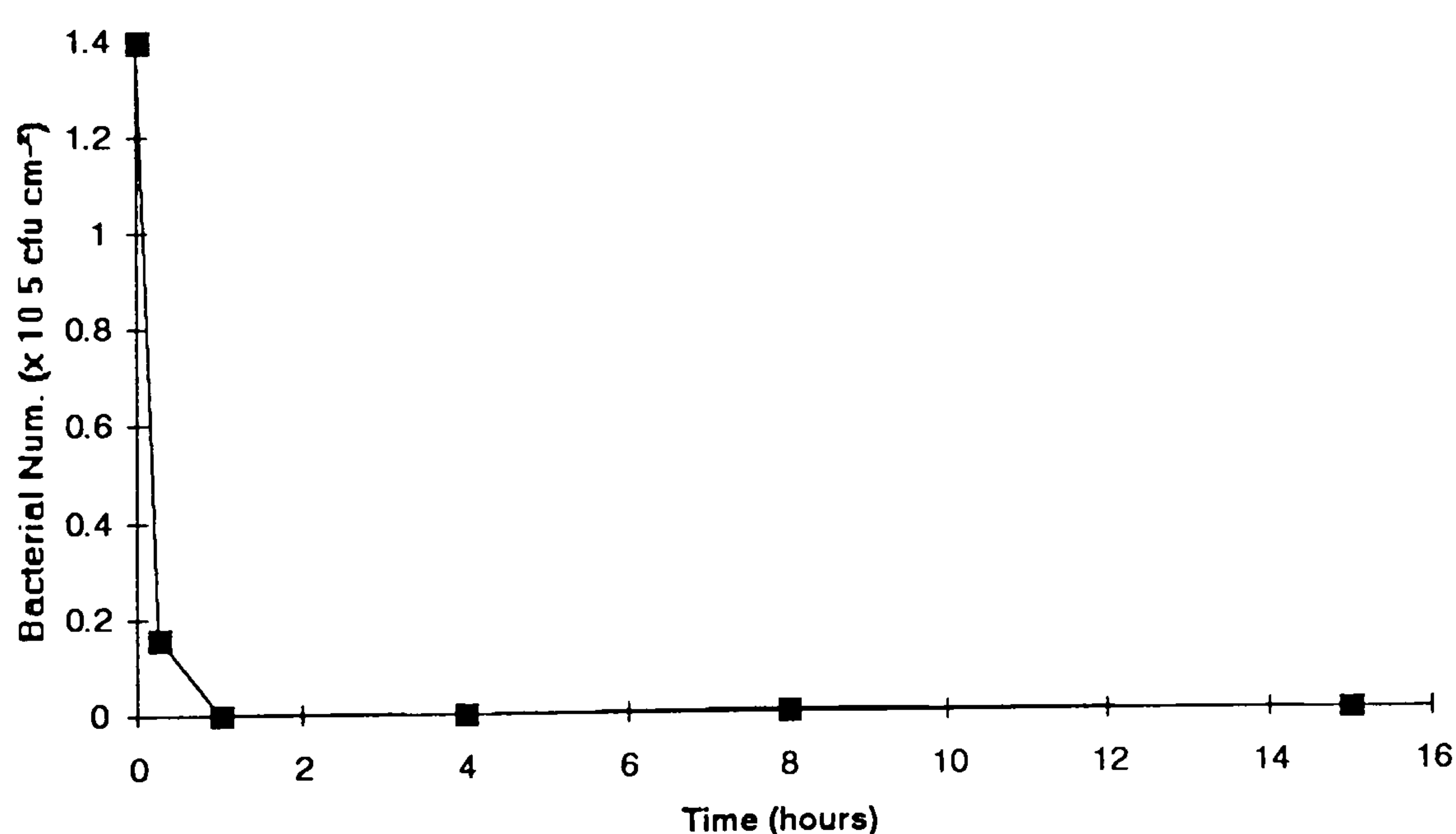


Figure 4.49 Pasteurisation at 60°C of mature biofilm established on copper at 45°C. Prior to pasteurisation 1.4×10^5 cfu cm^{-2} were recovered from copper surfaces matured in the chemostat for 42 days at 45°C. However after 15 minutes pasteurisation at 60°C, 1.6×10^3 cfu cm^{-2} were recovered, representing a 98.9 % decrease in recovery of biofilm bacteria. After 1 h pasteurisation a 99 % decrease in viability was achieved and was maintained for 15 days.

(i) Percentage profile of biofilm before and after pasteurisation

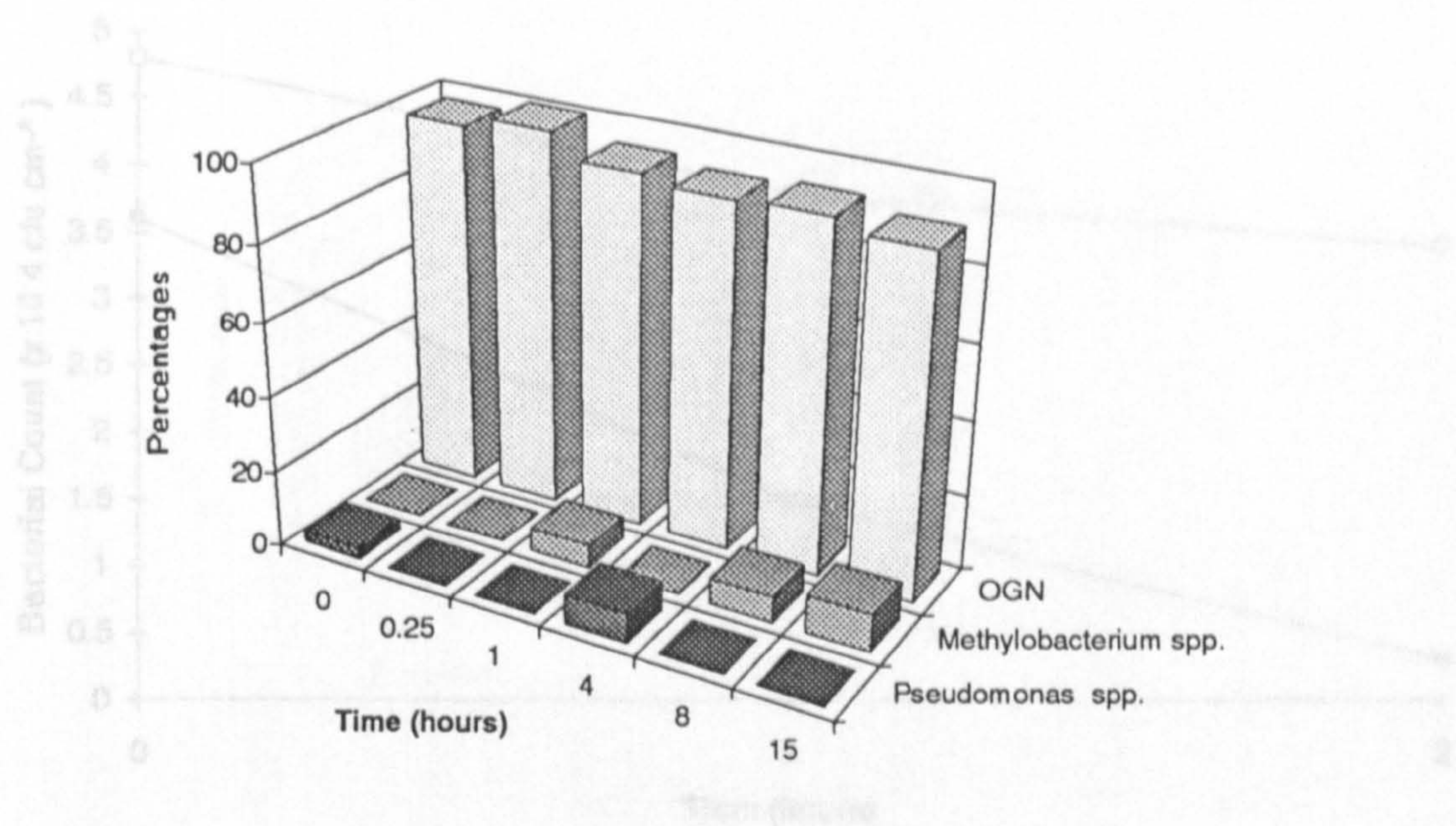


Figure 4.50 Percentage profile of biofilm matured at 45°C and pasteurised at 60°C.

Species / Time (h)	0	0.25	1	4	8	15
<i>Pseudomonas</i> spp.	3	0	0	8	0	0
<i>Methylobacterium</i> spp.	0	0	6	0	7	10
Gram negative bacteria	97	100	94	92	93	90

Table 4.28 Percentage profile of biofilm matured at 45°C and pasteurised at 60°C.

surfaces decreased to 1.5×10^4 cfu cm⁻² and 3.1×10^3 cfu cm⁻² after 1 h and 2 h

Prior to pasteurisation *Pseudomonas* spp. were present at 3 % and Gram negative bacteria at 97 % of the total population. After 15 min of exposure to 60°C only Gram negative bacteria were detected. *Methylobacterium* spp. were however recovered at 1, 8 and 15 h at 6 %, 7 % and 10 % respectively with *Pseudomonas* spp. also present at 8 % at 4 h.

in the vicinity to 3.7×10^4 cfu cm⁻² followed by a 25 % decrease of 25 %

to 3.6×10^4 cfu cm⁻² after 2 h pasteurisation.

4.6.1.1 Effect of Pasteurisation at 60°C on pre-established biofilm.

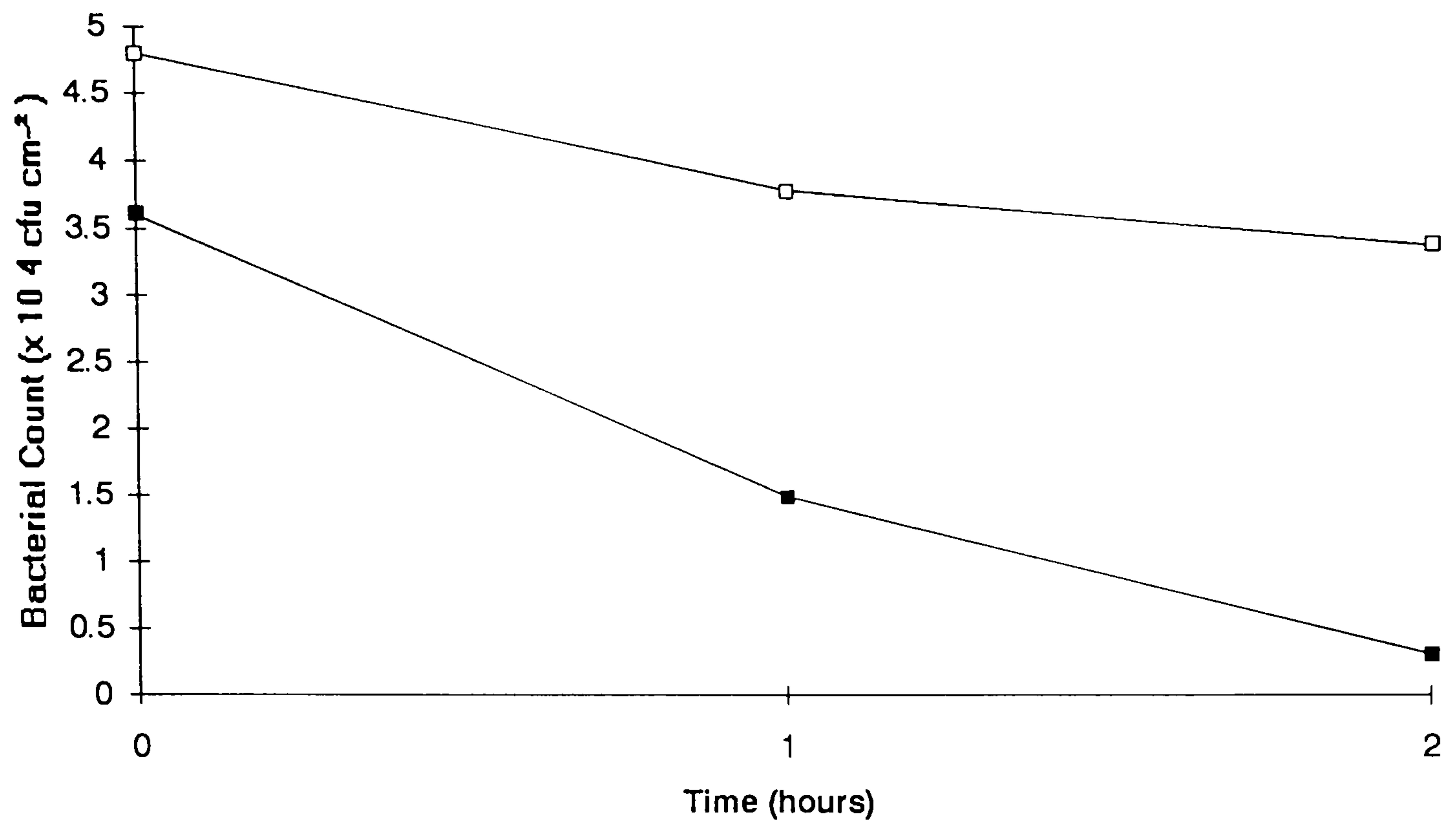


Figure 4.51 Pasteurisation at 60°C of mature biofilm established on copper and glass between 40-60°C. Biofilm recovered from copper and glass surfaces.

After 14 days maturation 3.6×10^4 cfu cm⁻² were recovered from the copper surfaces. Following pasteurisation at 60°C the number of bacteria recovered from the copper surfaces decreased to 1.5×10^4 cfu cm⁻² and 3.1×10^3 cfu cm⁻² after 1 h and 2 h respectively. This decrease in bacterial numbers represented a 59 % and 92 % reduction in viability of the biofilm after 1 h and 2 h respectively. The bacterial numbers recovered from glass surfaces were 4.7×10^4 cfu cm⁻² after 14 days maturation between 40-60°C. After pasteurisation at 60°C for 1 h there was a 22 % decrease in the viability to 3.7×10^4 cfu cm⁻² followed by a further decrease of 25 % to 3.6×10^4 cfu cm⁻² after 2 h pasteurisation.

(i) Percentage profile of pasteurised biofilm.

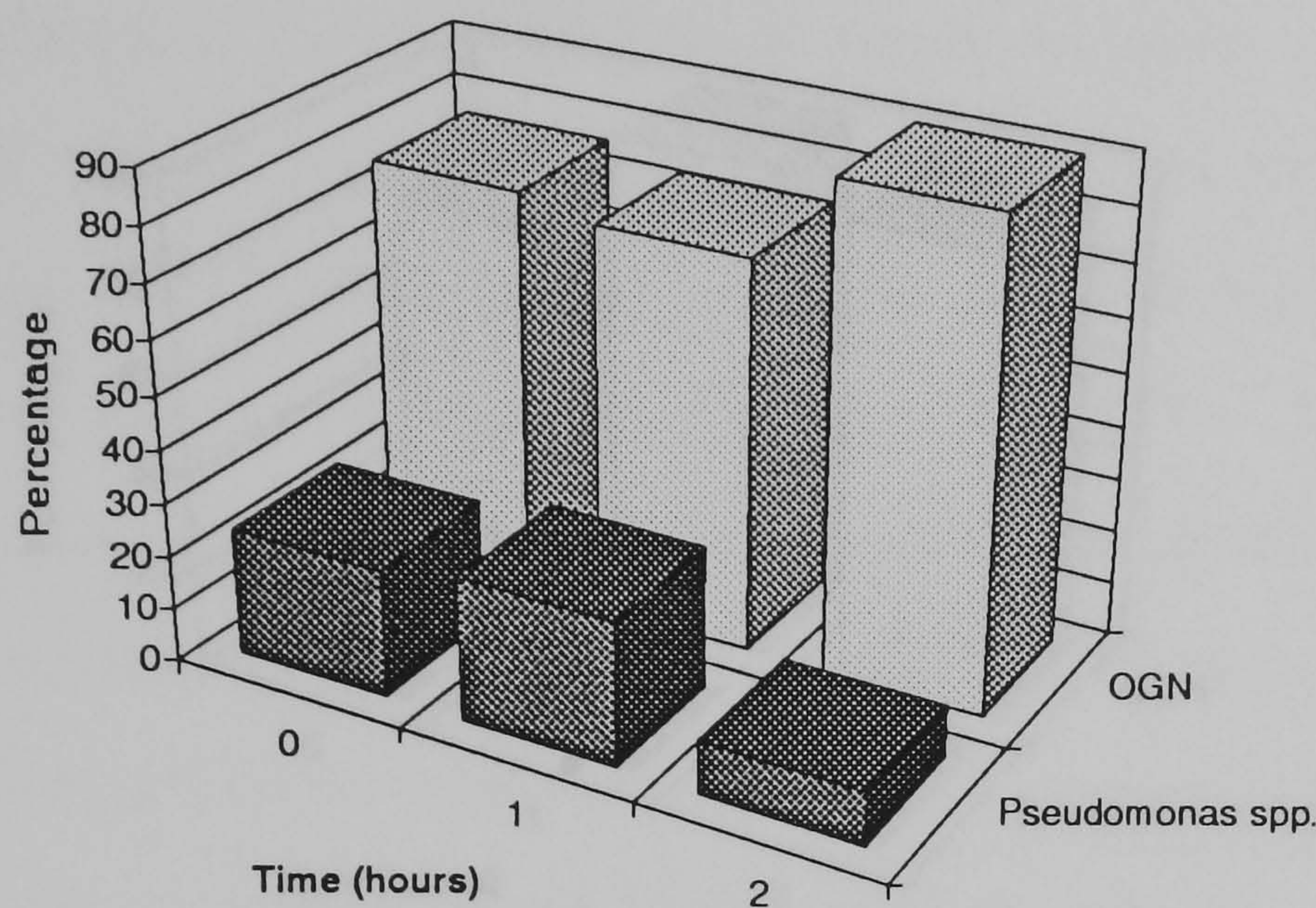


Figure 4.52 Percentage profile of bacteria on copper before and after pasteurisation at 60°C.

Species / Time (h)	0	1	2
<i>Pseudomonas spp.</i>	24	27	10
Gram negative bacteria	76	73	90

Table 4.29 Percentage profile of biofilm from copper before and after pasteurisation at 60°C.

Two bacterial types were recovered from the copper surfaces. The Gram negative bacteria dominated with the *Pseudomonas* spp. being recovered at 24 % of the population before pasteurisation. After 1 h pasteurisation the percentage profiles were still very similar even though the total numbers had been reduced. After 2 h at 60°C the *Pseudomonas* spp. were reduced to 10% of the population.

(ii) Percentage profile of pasteurised biofilm.

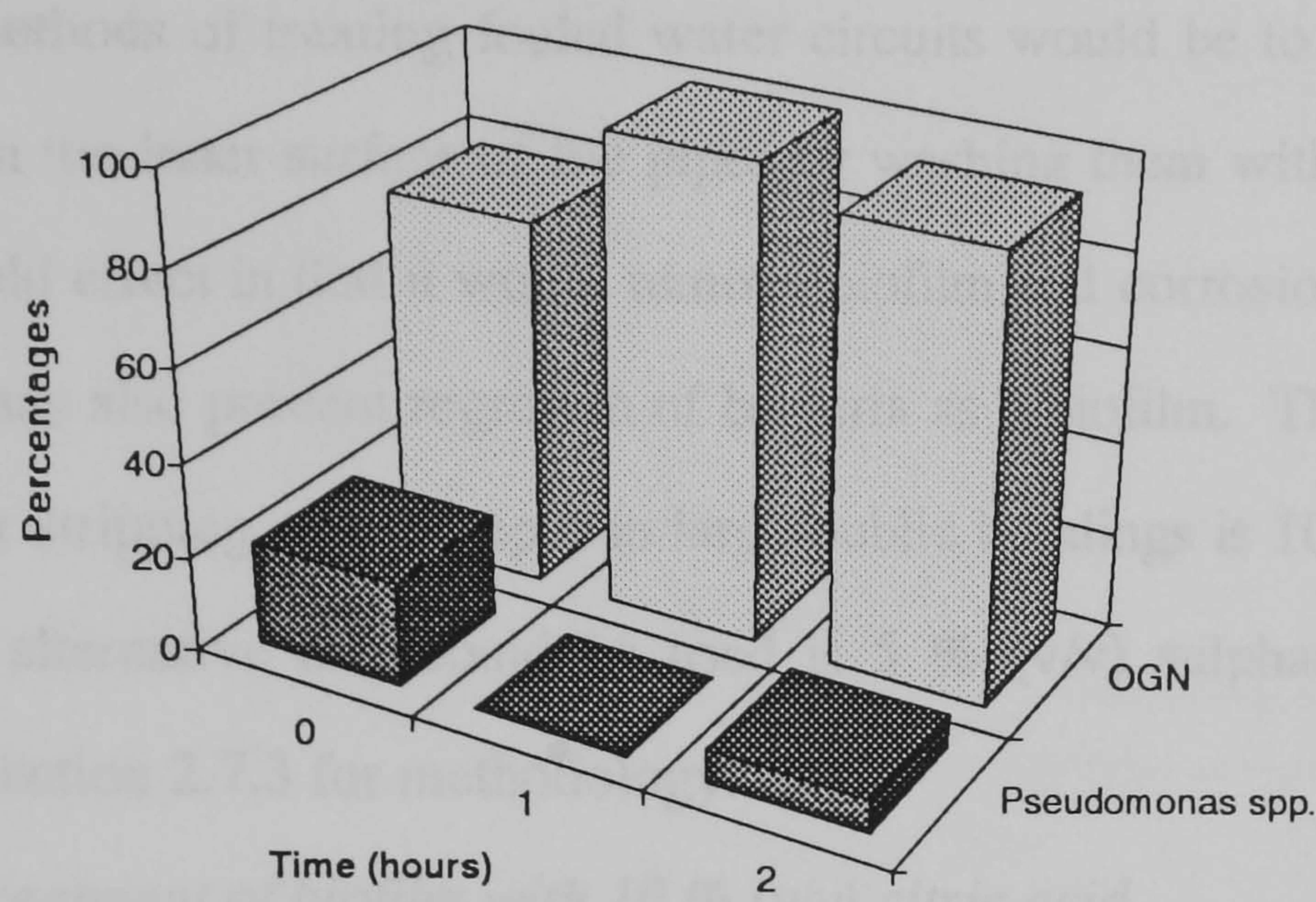


Figure 4.53 Percentage profile of bacteria on glass before and after pasteurisation at 60°C.

Species / Time (h)	0	1	2
<i>Pseudomonas spp.</i>	22	0	7
Gram negative bacteria	78	100	93

Table 4.30 Percentage profile of bacteria from glass before and after pasteurisation at 60°C.

Before pasteurisation *Pseudomonas* spp. were recovered at 22 % of the population but after pasteurisation the Gram negative bacteria dominated. No *Pseudomonas* spp. were recovered after 1 h pasteurisation. However after 2 h pasteurisation *Pseudomonas* spp. represented 7 % of the population.

4.6.2. Acid treatment of established biofilm

One of the methods of treating fouled water circuits would be to disrupt and remove any biofilm on the inner surface of the pipes by washing them with acids. This would have a two fold effect in that it would remove biofilm and corrosion products from the surface and may also prevent regrowth of bacteria as a biofilm. The only acid allowed to be used for stripping copper pipes in large public buildings is 10 % (v/v) citric acid. However an alternative that could be used is 5 % (v/v) sulphamic acid. Refer to Chapter 2.0 section 2.7.3 for methodology.

4.5.1.1 Treatment of biofilm with 10 % (v/v) citric acid.

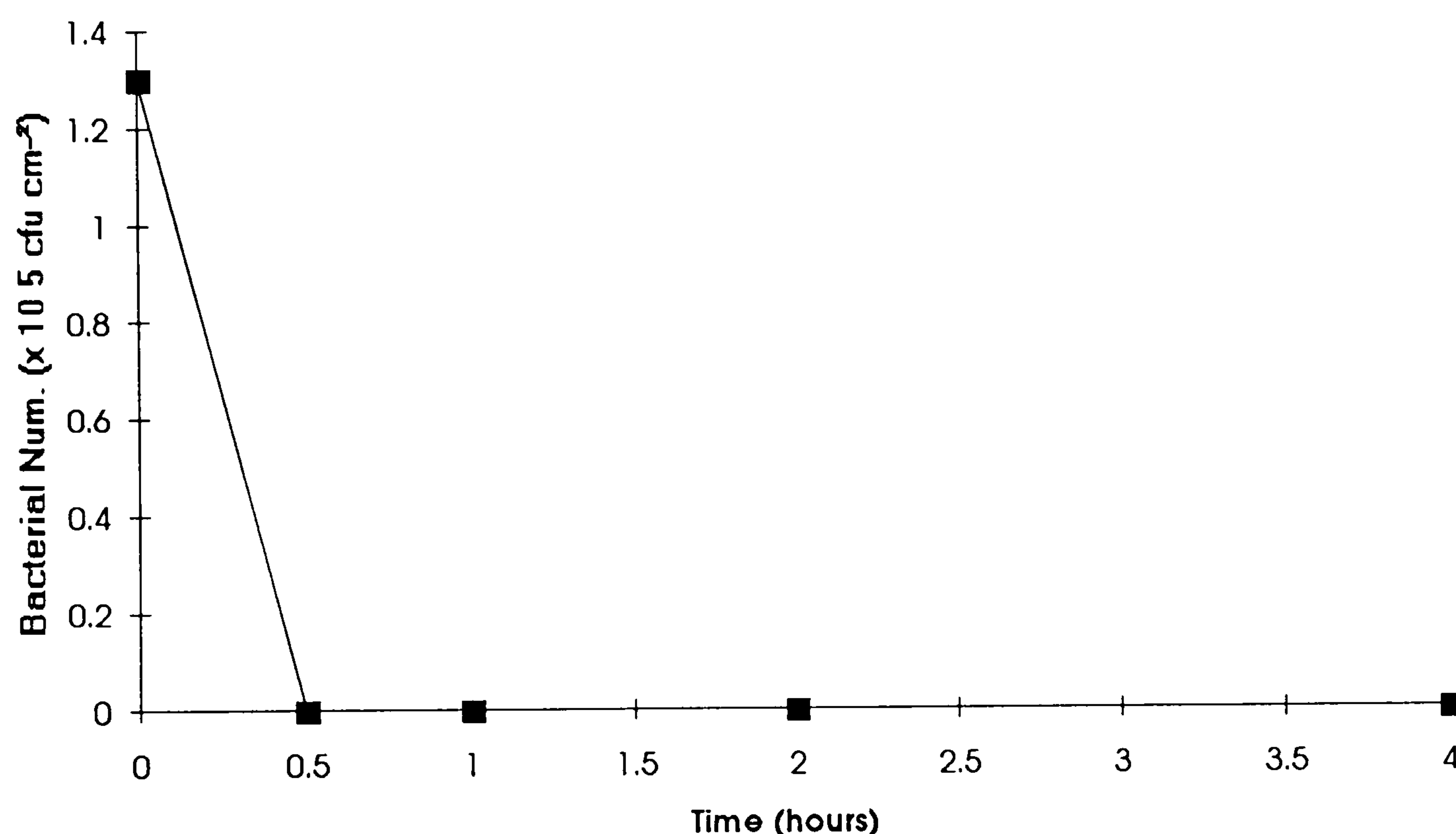


Figure 4.54 Citric acid (10 % v/v) treatment of 14 day old biofilms on copper matured at 45°C.

Prior to treatment with citric acid $1.3 \times 10^5 \text{ cfu cm}^{-2}$ were recovered from a 14 day old biofilm on copper. The biofilms were then exposed to 10 % v/v citric acid and after 30 min, $< 60 \text{ cfu cm}^{-2}$ were recovered. As there was greater than a 99 % inhibition after half an hour then this was chosen as the time period over which to treat mature biofilm before they were replaced back into the chemostat to study recolonisation.

(i) Percentage profile of bacteria before and after acid treatment.

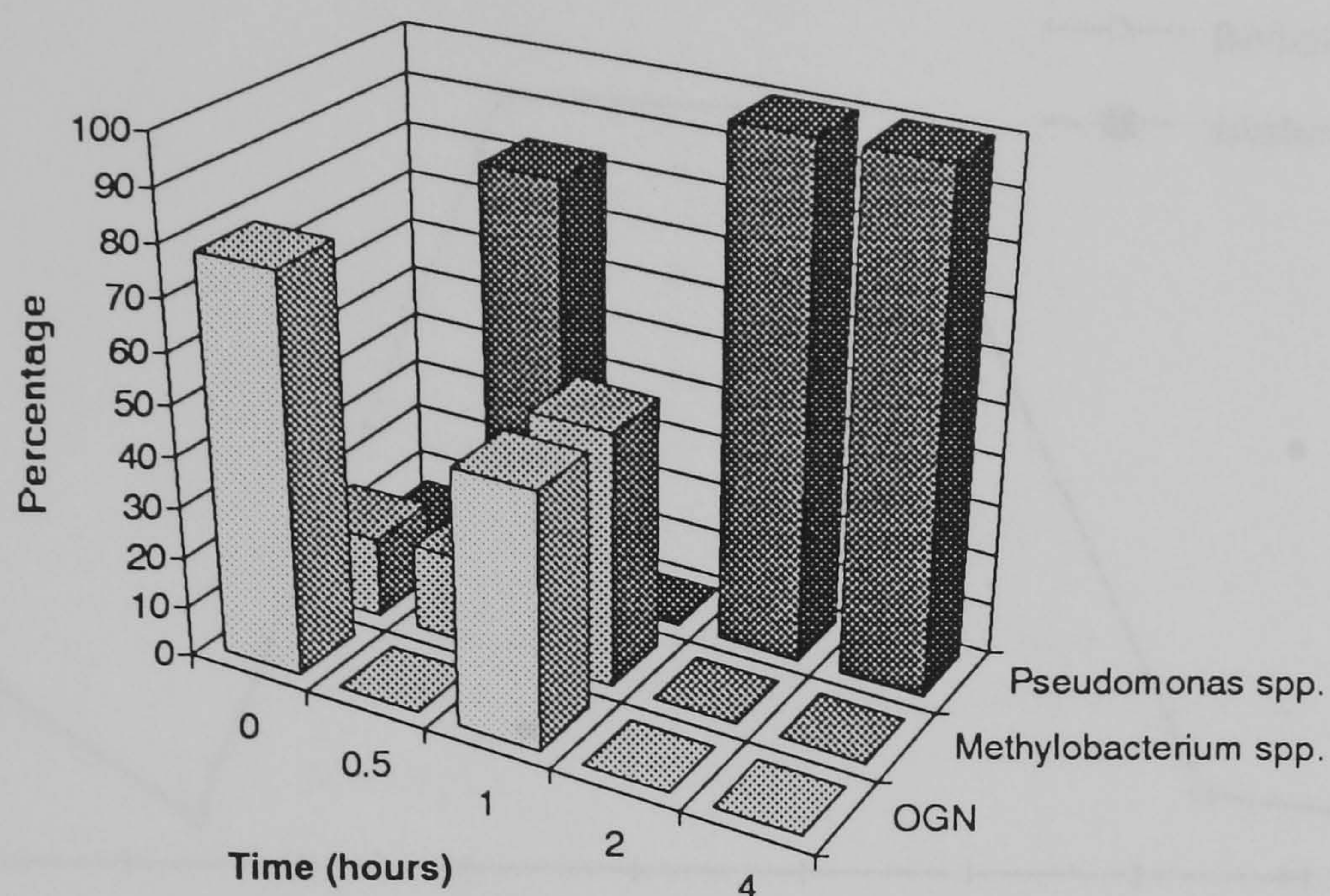


Figure 4.55 Percentage profile of copper surfaces before and after treatment with 10 % (v/v) citric acid.

Species / Time (h)	0	0.5	1	2	4
Gram negative bacteria	78	0	50	0	0
<i>Methylobacterium</i> spp.	16	17	50	0	0
<i>Pseudomonas</i> spp.	6	83	0	100	100

Table 4.31 Percentage profile of copper surfaces before and after treatment with 10 % (v/v) citric acid.

Prior to citric acid treatment the Gram negative bacteria dominated the biofilm at 78 % of the total population within the 14 day old consortium on the copper surface with the *Methylobacterium* spp. at 16 % and the *Pseudomonas* spp. representing 6 % of the total population. Following 0.5 hour treatment with citric acid *Pseudomonas* spp. dominated the biofilm.

4.6.2.2 Recolonisation of citric acid treated coupons

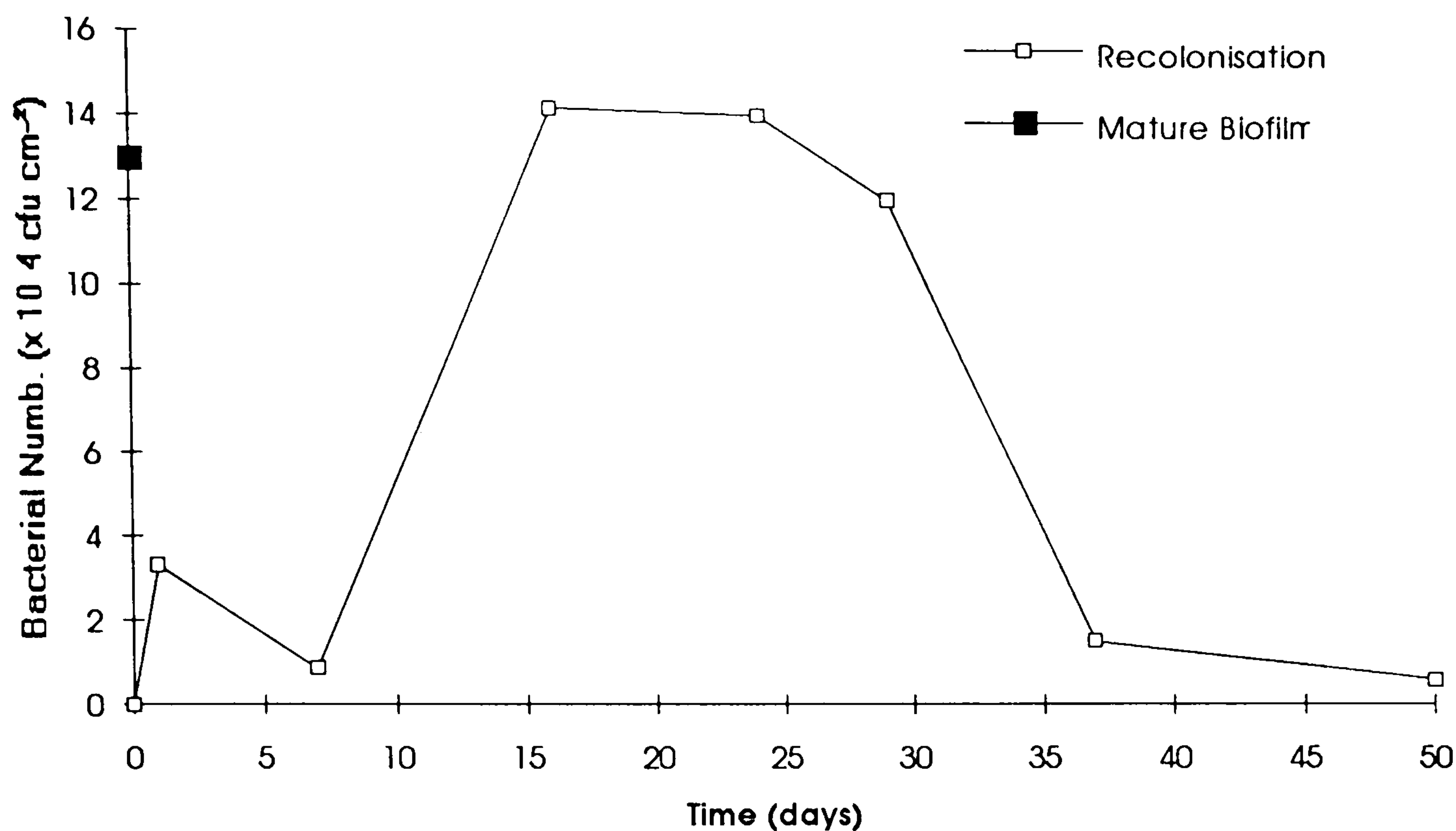


Figure 4.56 Recolonisation of copper coupons over 50 days following treatment for half an hour with 10 % v/v citric acid .

Prior to treatment with citric acid 1.3×10^5 cfu cm^{-2} were recovered from a 14 day old biofilm on copper. Following treatment with citric acid, 3.3×10^4 cfu cm^{-2} were recovered from the copper coupons with only 8.5×10^3 cfu cm^{-2} at day 8. From day 16 to 29, $> 1.2 \times 10^5$ cfu cm^{-2} were recovered from the copper surfaces. However, by day 37 there was a decrease in the number of bacteria recovered to 1.5×10^4 cfu cm^{-2} with 6.0×10^4 cfu cm^{-2} at day 50.

(i) Percentage profile of citric acid treated coupons.

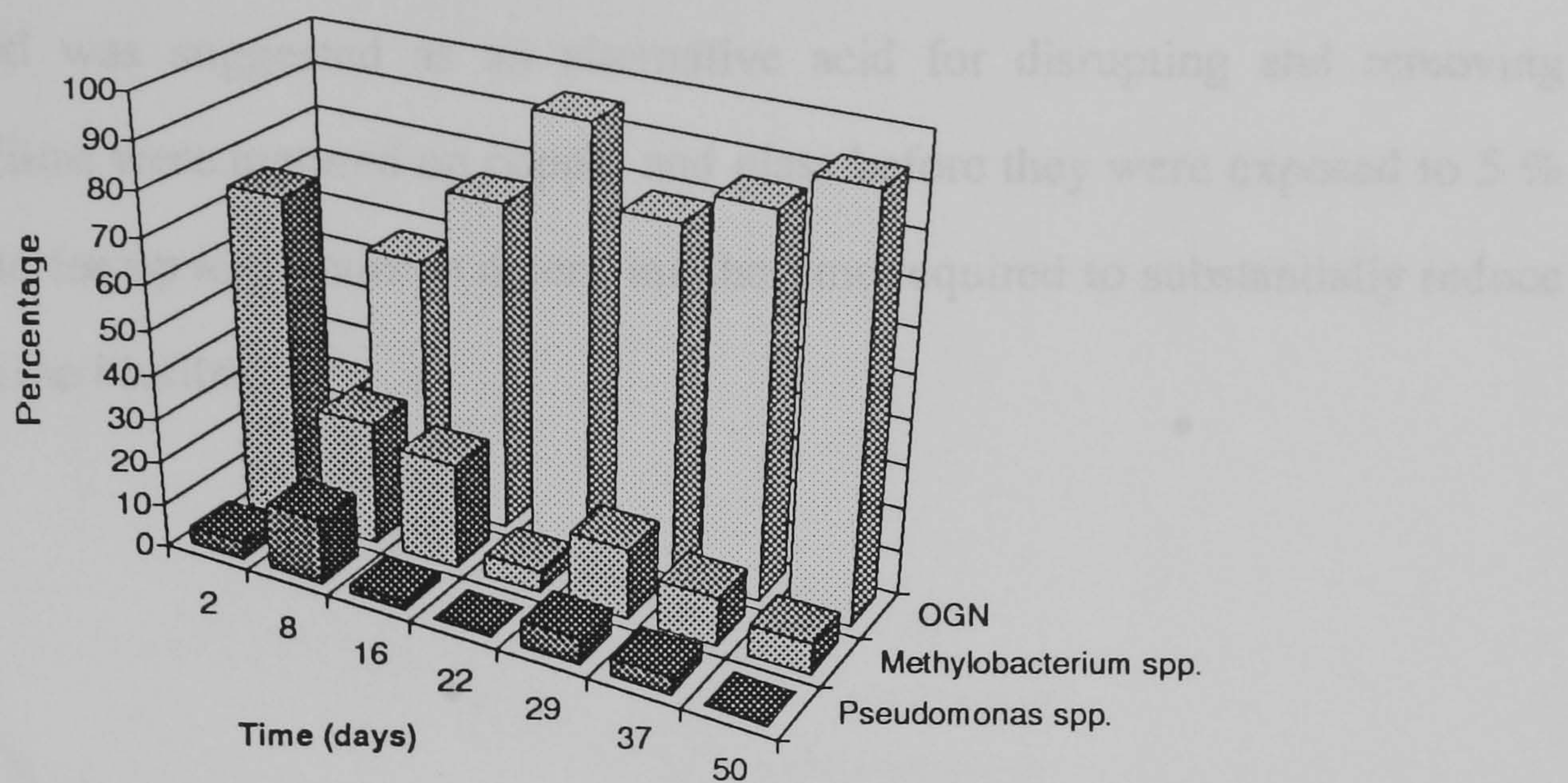


Figure 4.57 Percentage profile from citric acid treated copper coupons recolonised over 50 days.

Species / Time (days)	2	8	16	22	29	37	50
<i>Pseudomonas</i> spp.	4	15	2	0	6	4	0
<i>Methylobacterium</i> spp.	74	28	24	5	16	11	7
Gram negative bacteria	22	57	74	95	78	85	93

Table 4.32 Percentage profile from citric acid treated copper coupons recolonised for 50 days.

The Gram negative bacteria steadily increased over the 50 days representing 80 % or more after day 22. In contrast the *Methylobacterium* spp. were dominant within the first 24 hours 74 % of the population but then declined in numbers as a proportion of the population. The *Pseudomonas* spp. only represented less than 10 % of the population.

4.6.2.3 Treatment of biofilms with sulphamic acid.

Sulphamic acid was suggested as an alternative acid for disrupting and removing biofilms. Biofilms were matured on copper and glass before they were exposed to 5 % (v/v) sulphamic for up to 2 hours to determine the time required to substantially reduce the viability of the biofilm.

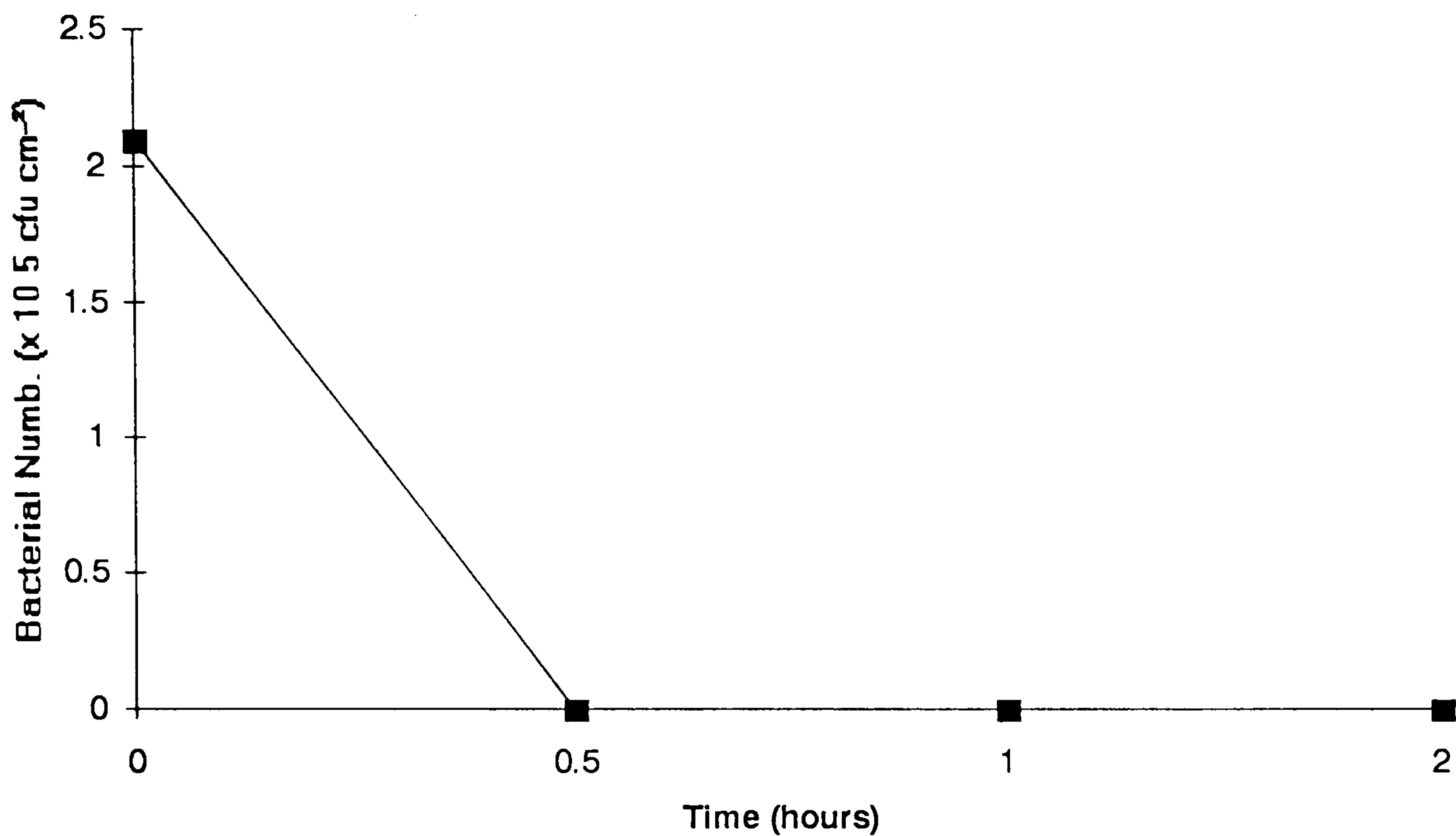


Figure 4.58 Sulphamic acid (5 % v/v) treatment of 14 day old biofilms on copper established at 45°C.

A total of 2.1×10^5 cfu cm⁻² were recovered from 14 day old biofilms. However, only half an hour treatment with 5 % (v/v) sulphamic acid was required to achieve a complete reduction in the viability of the biofilm. Therefore when studying the effect of sulphamic acid on the recolonisation of copper coupons they were first treated with 5 % (v/v) sulphamic acid for half an hour prior to being replaced in the chemostat.

(i) Percentage profile of bacteria treated with sulphamic acid.

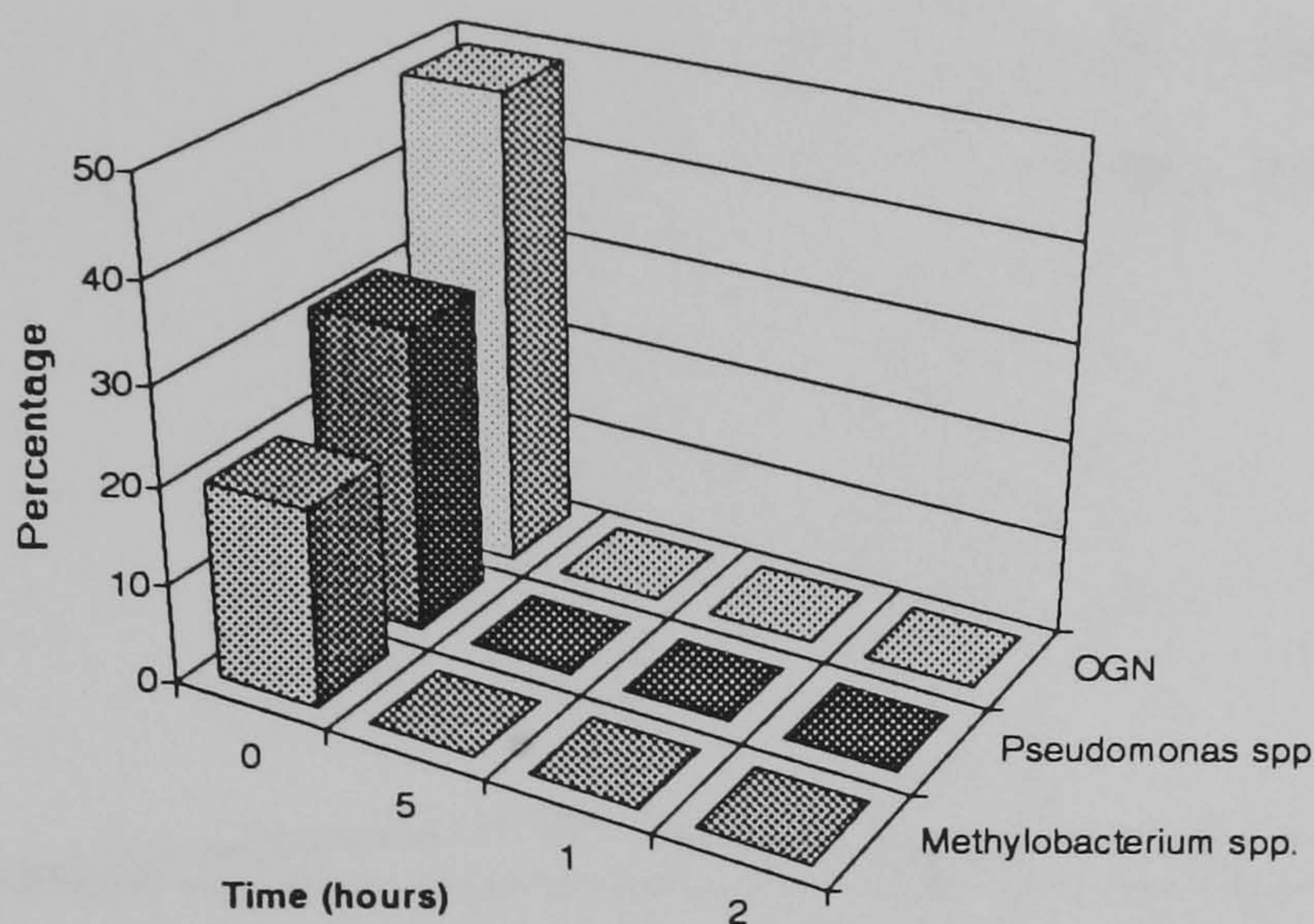


Figure 4.59 Percentage profile of sulphamic acid treated biofilms on copper established at 45°C.

Species / Time (h)	0	5	1	2
Methylobacterium spp.	20	0	0	0
Pseudomonas spp	31	0	0	0
Gram negative bacteria	49	0	0	0

Table 4.33 Percentage profile of sulphamic acid treated biofilms on copper established at 45°C.

The population from the 14 day old biofilm composed of 49 % Gram negative bacteria, 31 % *Pseudomonas* spp. and 20 % *Methylobacterium* spp. however, no species were detected after half an hour treatment with sulphamic acid.

4.6.2.4 Recolonisation of sulphamic acid treated coupons.

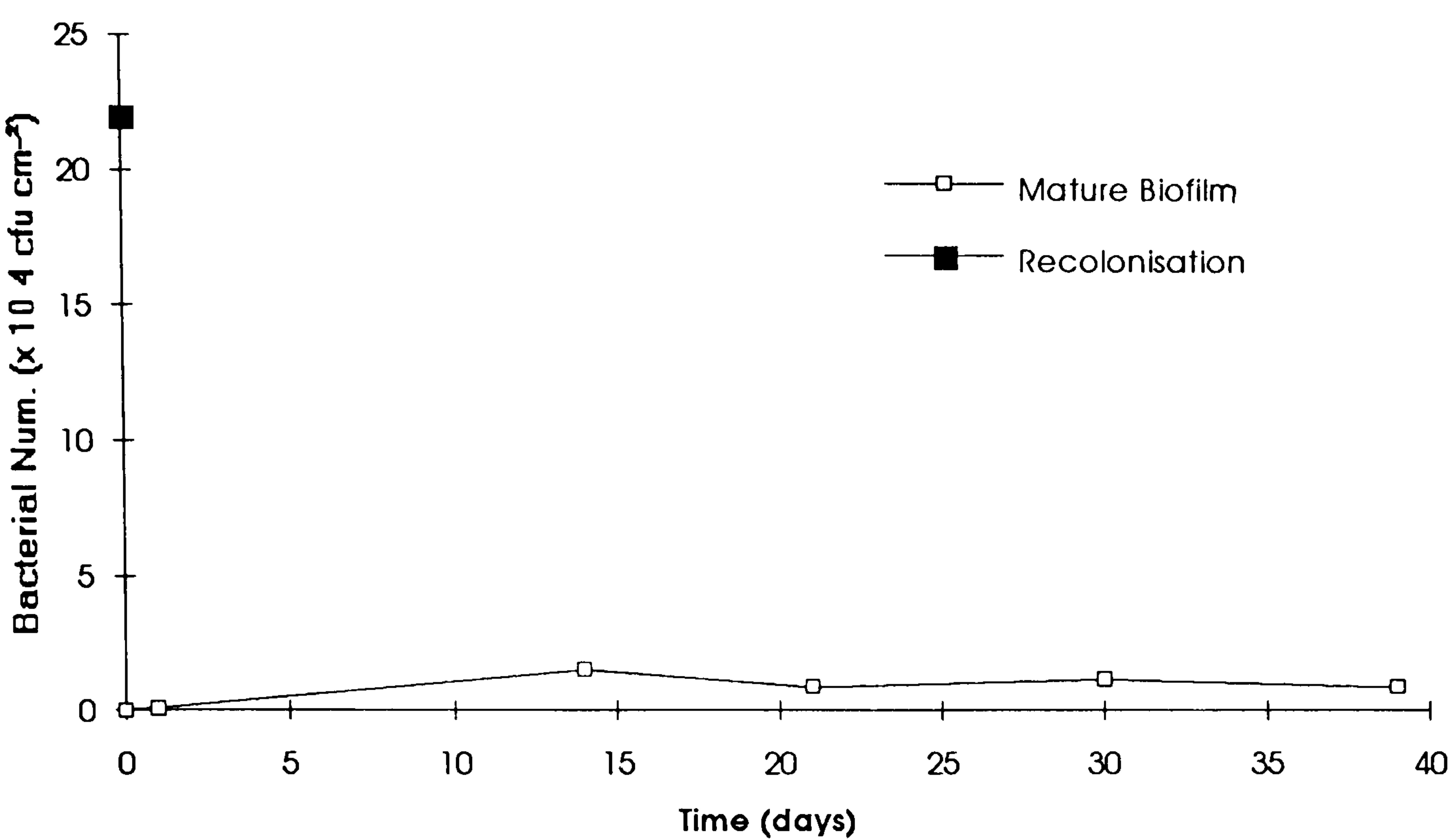


Figure 4.60 Recolonisation of copper coupons for 39 days after being treated for half an hour with sulphamic acid.

Recolonisation of the sulphamic acid treated coupons occurred at 855 cfu cm⁻² in 24 hours but by day 14 this had risen to 1.5 x 10⁴ cfu cm⁻². At day 21 only 8.7 x 10³ cfu cm⁻² were recovered from the treated coupons but by day 30 this had increased to 1.1 x 10⁴ cfu cm⁻² and decreased to 8.7 x 10³ cfu cm⁻² at day 39.

(i) Percentage profile of bacteria treated with sulphamic acid.

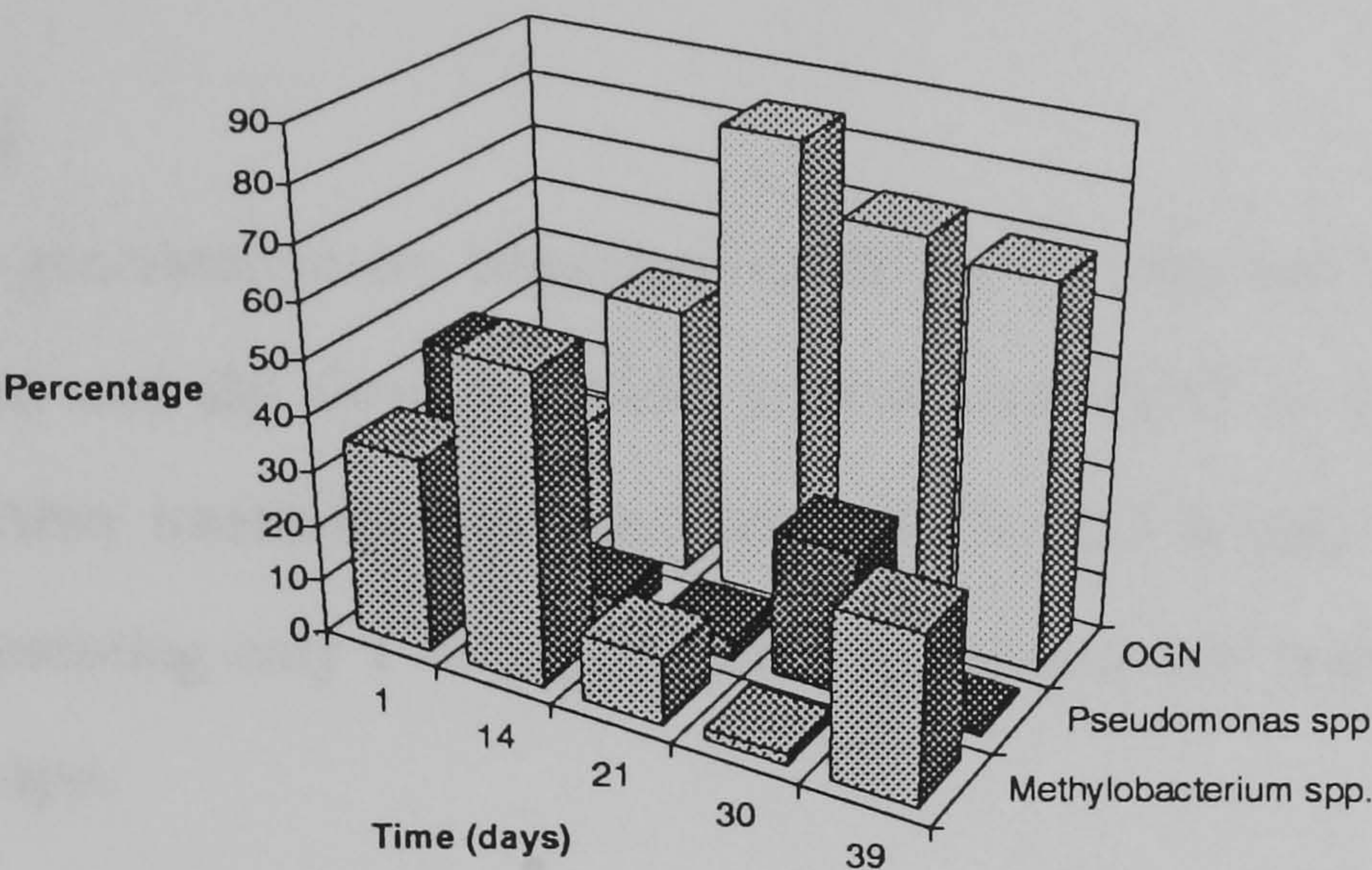


Figure 4.61 Percentage profiles of copper coupons recolonised for 39 days which were initially treated for half an hour with sulphamic acid.

Species / Time (days)	1	14	21	30	39
<i>Methylobacterium</i> spp.	35	56	12	2	30
<i>Pseudomonas</i> spp.	44	5	4	26	1
Gram negative bacteria	21	49	84	72	69

Table 4. 34 Percentage profile of copper coupons recolonised for 31 days which were initially treated for half an hour with sulphamic acid.

Primarily the *Pseudomonas* spp. dominated as the initial colonisers at day 1 at 44% of the population but steadily decreased in numbers as a proportion of the population. However the Gram negative bacteria steadily increased representing 84 % at day 21. Although the *Methylobacterium* spp. dominated at day 14 the numbers decreased thereafter as a proportion of the population.

(ii) In Summary

Citric Acid

Biofilms were generated in the laboratory model for 14 days and 1.3×10^5 cfu cm⁻² were recovered and the Gram negative bacteria dominated as a proportion of the population. After treatment with the citric acid for 0.5 h only 60 cfu cm⁻² were detected, representing only 1 % of the biofilm population and were dominated by the *Pseudomonas* spp.

Biofilms were established for 14 days at 45°C, extracted from the chemostat and treated with citric acid for 15 min (as this time period had eradicated 99 % of the viable biofilm) before being re-immersed into the chemostat at 45°C. Recolonisation was followed for 50 days (Fig. 4.56). Before citric acid treatment 1.39×10^5 cfu cm⁻² recovered at day 14 however, less than 3.3×10^4 cfu cm⁻² were recovered up to day 8. From day 14 to day 29 more than 1.0×10^5 cfu cm⁻² were recovered from the copper coupons. At day 37 the number of bacteria recovered decreased by then exhibited an increase to 60×10^3 cfu cm⁻² at day 50. As far as the population profiles were concerned the *Methylobacterium* spp. dominated immediately after treatment with citric acid but then declined as the Gram negative bacteria steadily increased.

4.8.5.iv Sulphamic Acid

Sulphamic acid was chosen as an alternative to citric acid. However using 5 % (v/v) sulphamic acid the viability of bacteria in the biofilm was completely reduced (Fig. 4.58). As with the biofilm removed from the culture for citric acid treatment the population was dominated by the Gram negative bacteria with the *Pseudomonas* spp. and *Methylobacterium* spp. also present. The recolonisation of copper coupons immersed in sulphamic acid (5 % v/v) for 0.5 h can be observed in (Fig. 4.60). Plotted at zero on the x-axis is the 210×10^3 cfu cm⁻² recovered for the 14 day old biofilm before it was treated with sulphamic acid. At 14 days only 15×10^3 cfu cm⁻² were

recovered from the copper coupons which was a maximum for the 40 day recolonisation. Species diversity within the biofilm was dominated by the *Pseudomonas spp.* within 24 and the *Methylobacterium spp.* at day 14 but thereafter the Gram negative bacteria steadily increased until day 50.

4.7 INFLUENCE OF WATER CHEMISTRY ON BIOFOULING

Calcium carbonate concentration was increased from < 20 ppm in the soft water in the media supplying only vessel two such that this vessel had a final working concentration of 70-80 ppm.

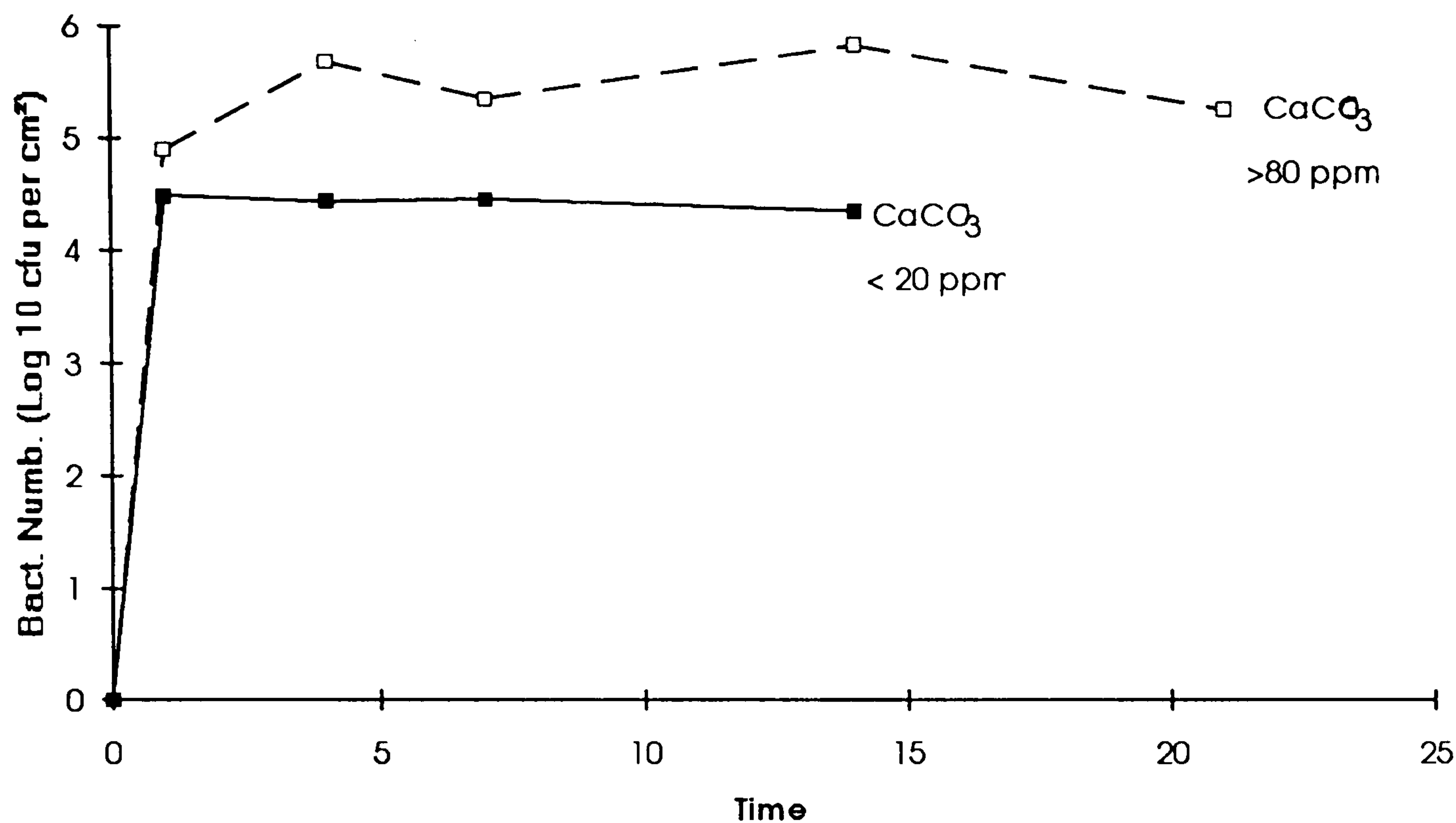


Figure 4.31 Colonisation of copper before and after additional CaCO₃.

Initially in the unaltered soft water the colonisation of copper occurred at 4.49 Log 10 cfu cm⁻² at day 1 and 4.44, 4.47 and 4.38 Log 10 cfu cm⁻² at day 4, 7, and 14 respectively. Correspondingly when the concentration of calcium carbonate was increased to 80 ppm in the culture the number of viable bacteria recovered from the biofilm in 24 h was 4.9 Log 10 cfu cm⁻² with 5.7 Log 10 cfu cm⁻² at day 4. The number of bacteria recovered from the biofilm then decreased to 5.3 Log 10 cfu cm⁻² at day 21.

4.8 VISUALISATION OF COPPER SURFACES (ESEM)

4.8.1 Influence of particulate matter on biofouling

The principle of environmental SEM is the visualisation of specimens without prior preparation. Hydrated specimens were loaded onto the stage and the chamber adjusted to a pressure of 7.0 torr. Subsequently as the pressure in the chamber was decreased water present on the surface was sublimed off to reveal the morphology of the specimen below. In Fig. 4.32 the presence of bacteria and debris on the surface are clearly visible as is a small area of copper surface. Image analysis revealed that the average area covered was equivalent to 32.3 % with 600 objects being identified (Table 1). The bacteria are observed in greater detail in Fig. 4.33 where the microcolony of biofilm and debris can be seen to be physically open with 5 μm spaces present which may form a series of water channels.

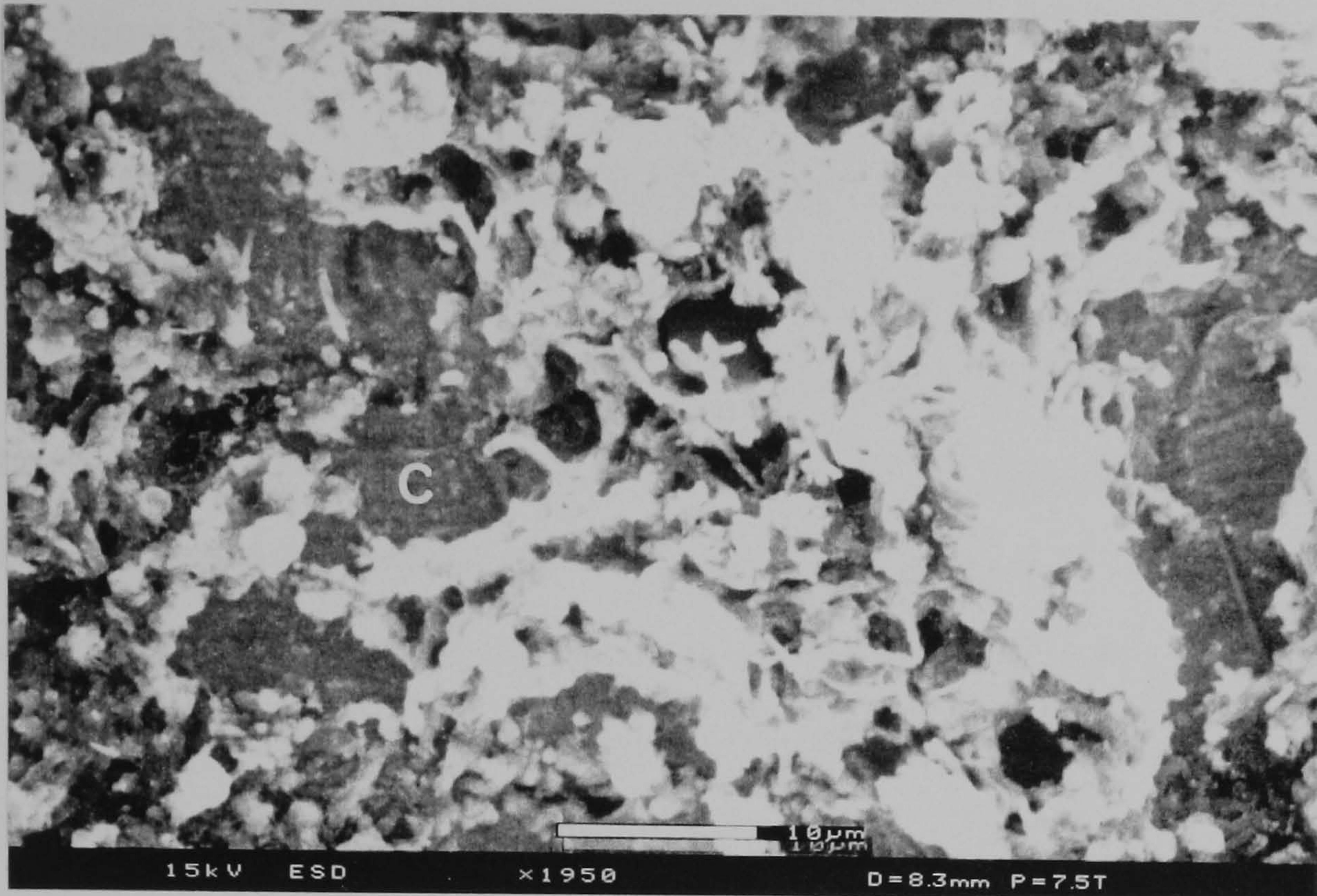


Figure 4.32 ESEM of copper coupon surface from culture supplied with particulate matter (marker bar denotes 10 μm). C denotes copper surface.

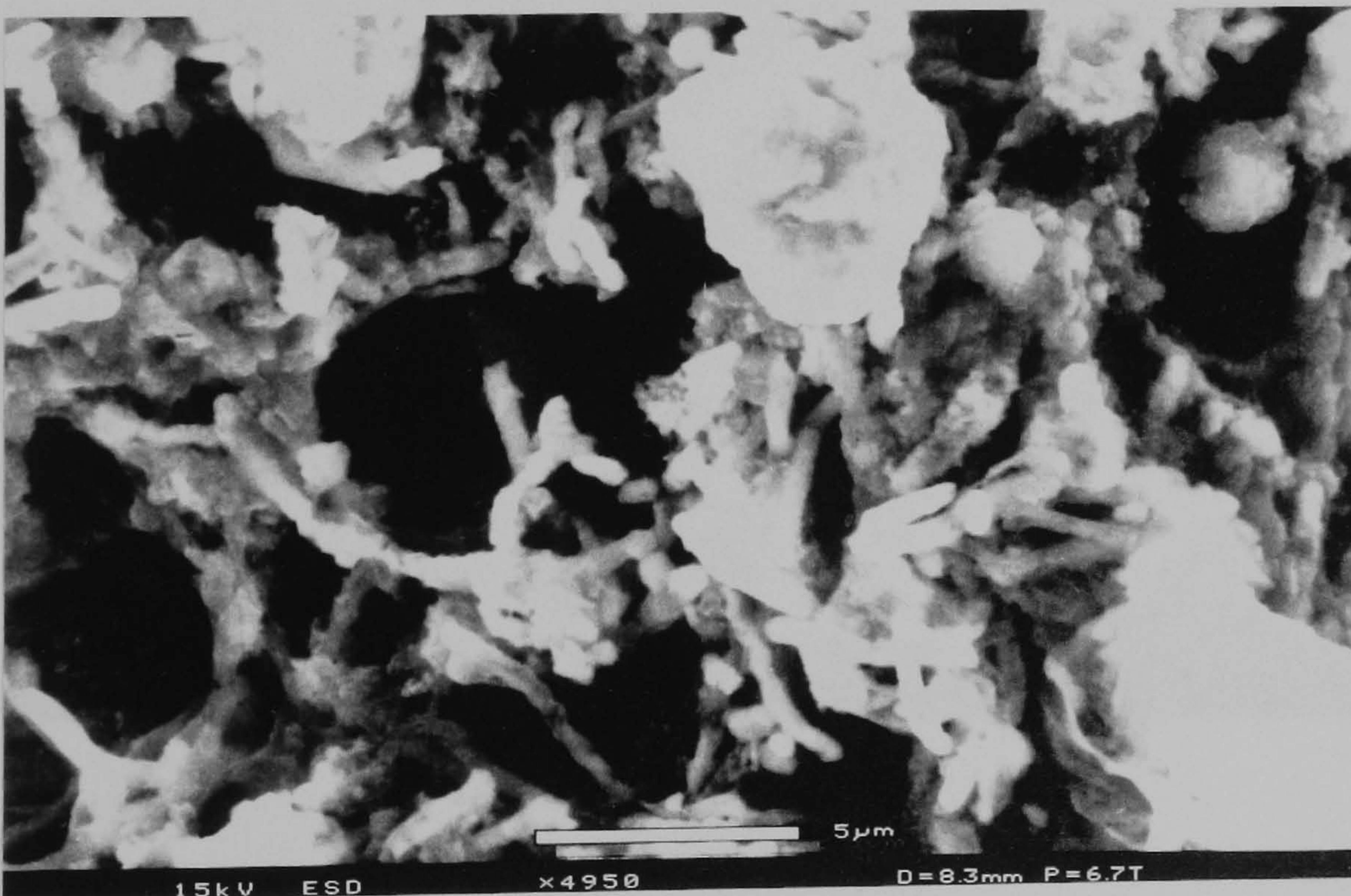


Figure 4.33 ESEM of copper coupon surface from culture supplied with particulate matter (marker bar denotes 5 μm).

4.8.2 Biofouling in the absence of particulate matter

The surface of the copper tile is relatively free from debris as the original milling lines are still visible Fig. 4.34. Although a microcolony of bacteria is visible there is very little particulate matter and debris on the surface. When the image was analysed the total percentage area covered was 29.8 % with 147 identifiable objects. At increased magnification, Fig. 4.35 bacteria are observed in the crevices in the copper surface. The only appreciable amount of debris found did not resemble particulate matter as it was too smooth and consistent and may have been tissue which attached to the tile surface during transportation, Fig. 4.36. However either side of this debris the naked copper surface is clearly visible.

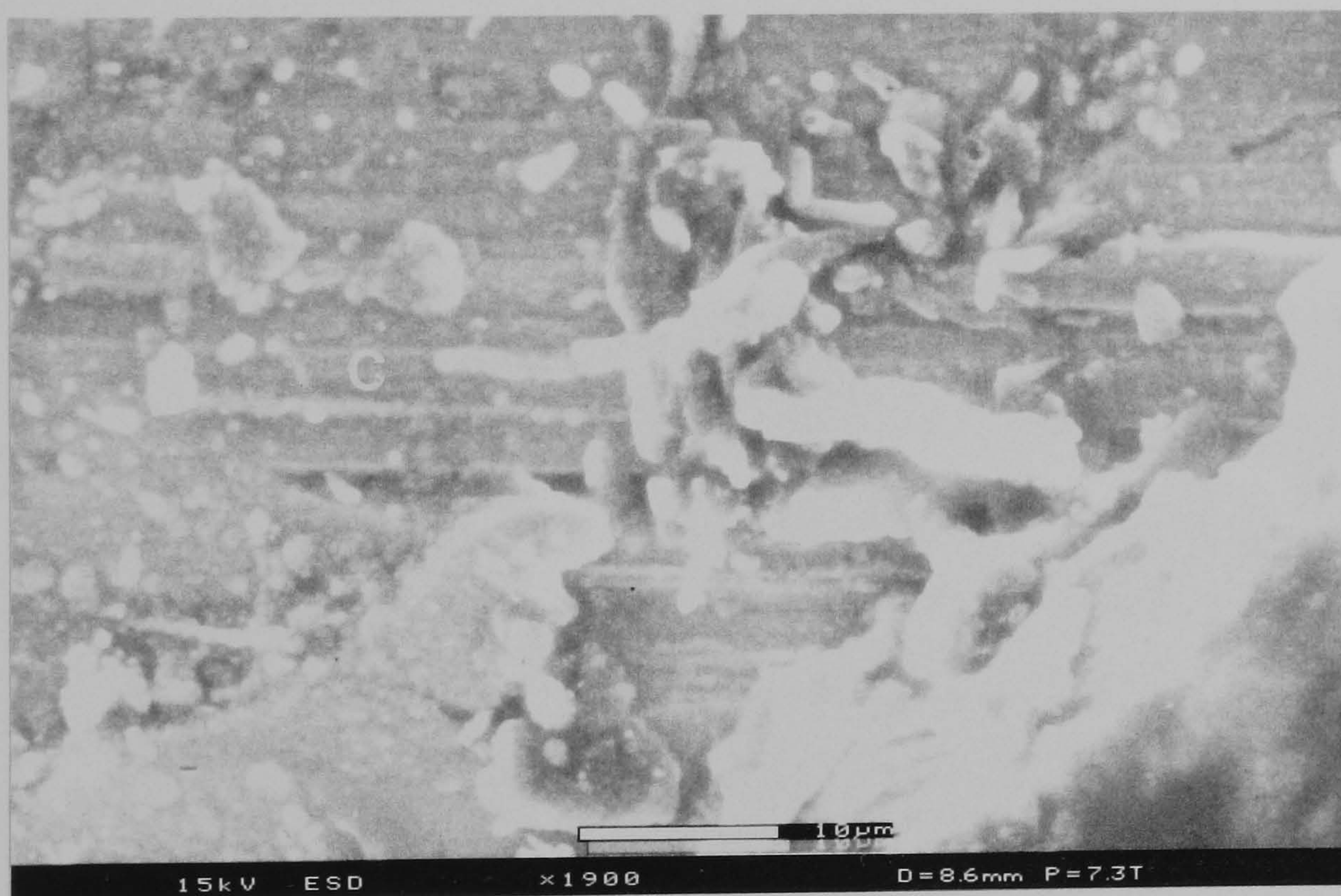


Fig. 4.34 ESEM of copper coupon surface from filtered culture (marker bar denotes 10 μm). C denotes the copper surface.

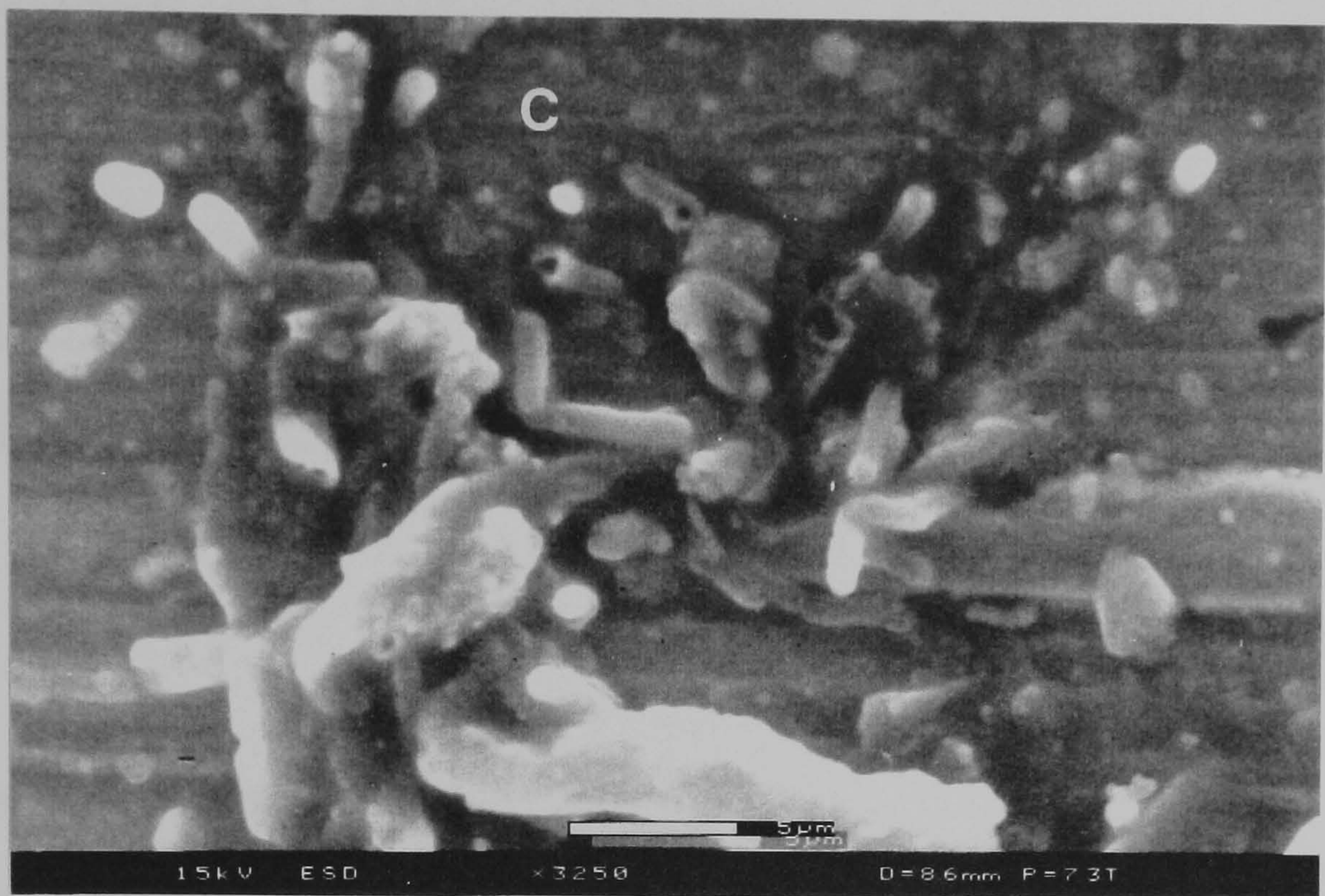


Figure 4.35 ESEM of copper coupon surface from filtered culture (marker bar denotes $5\ \mu\text{m}$). C denotes the copper surface.



Figure 4.36 Naked copper coupon surface as observed by ESEM from filtered culture (marker bar denotes $10\ \mu\text{m}$). C denotes the copper surface.

Table 4.9 ESEM area 1. Analysis of the area visualised by ESEM.

Field evaluation	Average (Fig. 1)	Average (Fig. 3)
% Area	32.3	29.8
Counted objects	600	147

4.8.3 Biofouling in the presence of particulate matter

The surface of the copper tube was covered completely in bacteria Fig. 4.37 (marker bar denotes 5 μm). The bacterial flora are relatively dense with the top of a hydrated microcolony clearly visible. No naked copper surface was visible in this specimen.

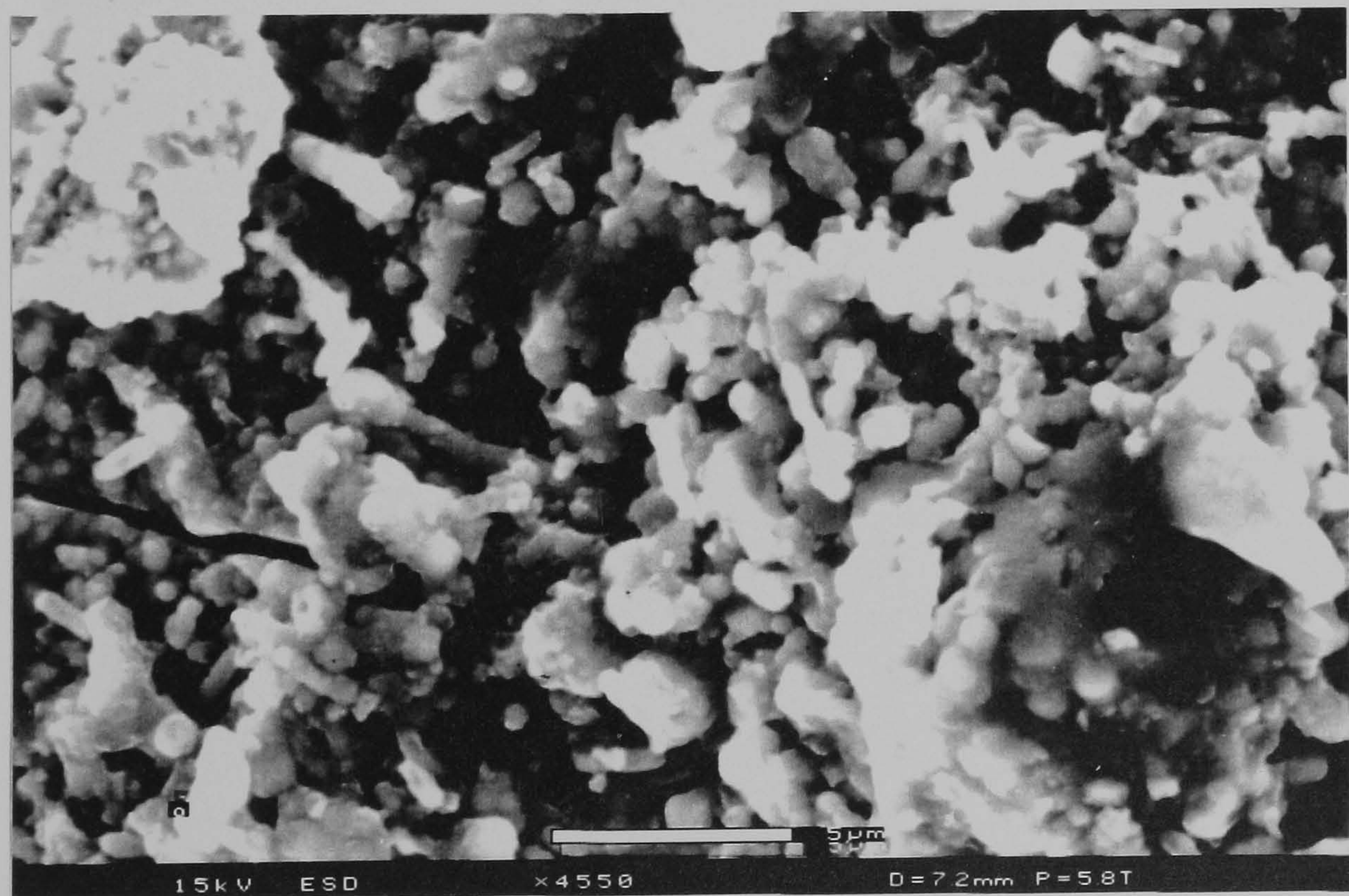


Fig. 4.37 ESEM of copper tube surface supplied with culture containing particulate matter (marker bar denotes 5 μm).

4.8.4 Scanning confocal laser microscope (SCLM)

4.8.4.1 Choice of coupons for SCLM analysis

During transportation, the coupons in the universals appeared to have lost part of the biofilm resulting in debris in the bottom of the universal. The hydrated coupons, retained in moist tissues in the petri dish, did not appear to suffer from detachment of appreciable amounts of biofilm and so were chosen for analysis.

4.8.4.2 Comparison of surface topology

Fluorescein was utilised to visualise the topology of the copper surfaces. The surface of copper coupons from the vessel supplied with filtered soft water exhibited a uniform and smooth surface morphology as indicated by the unchanging fluorescence over the surface (Fig. 4.38). When viewed in the horizontal (xz sagittal sectioning) mode the surface was also uniform (Fig. 4.39).

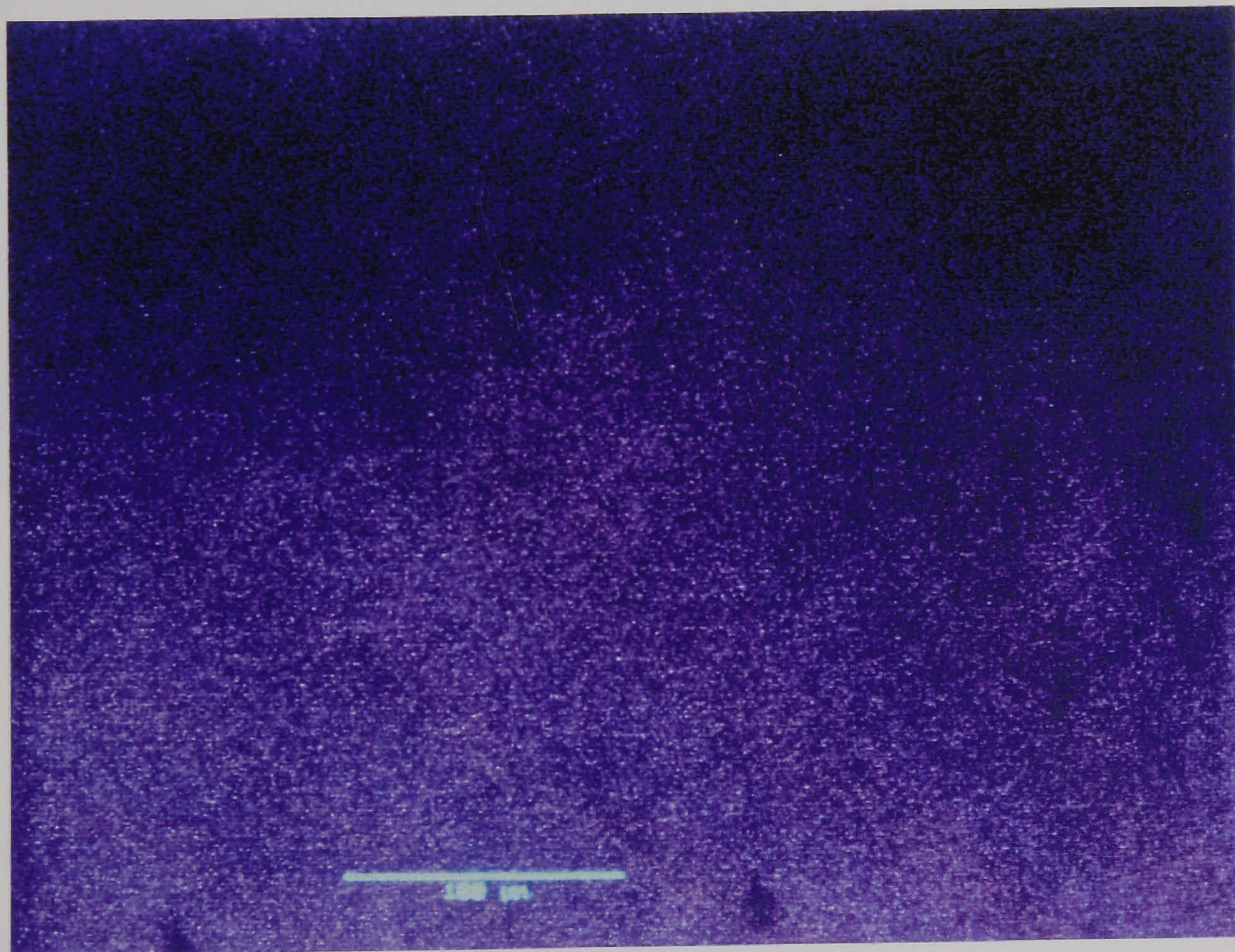


Fig. 4.38 Direct view of copper surface supplied with filtered soft water (marker bar denotes 100 μm).

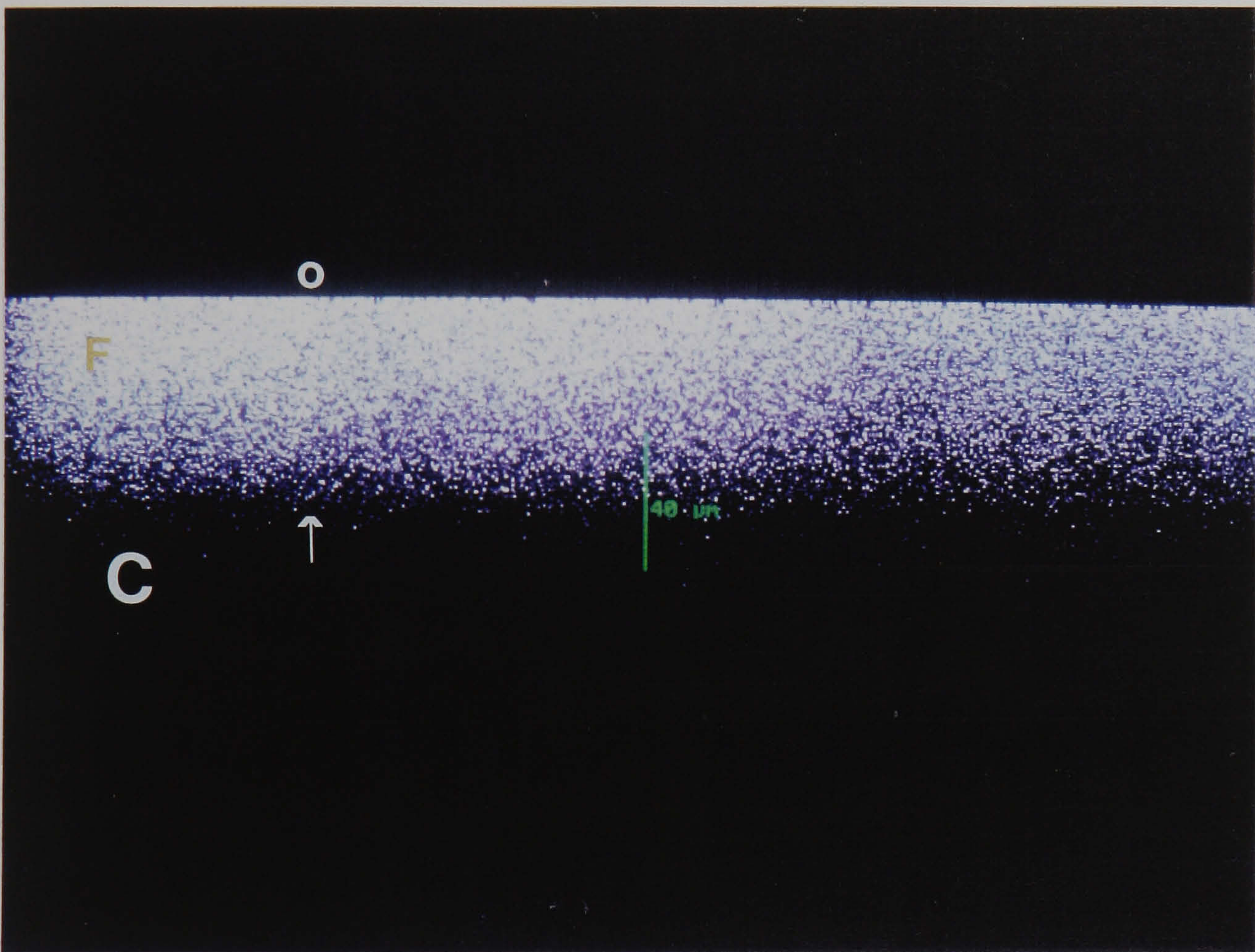


Fig. 4.39 An xz sagittal sectioning of copper surface supplied with filtered soft water (marker bar denotes $40\ \mu\text{m}$). F denotes the fluorescein on top of C the uniform copper surface with the arrow indicating the inner surface of the copper tube and O indicating the outer surface.

Copper coupons extracted from the vessel supplied with particulate matter exhibited an uneven surface with many peaks and troughs as indicated by the patchy fluorescence pattern (Fig. 4.40). An xz sagital through the surface revealed pits of up to $40\text{ }\mu\text{m}$ in depth (Fig. 4.41).

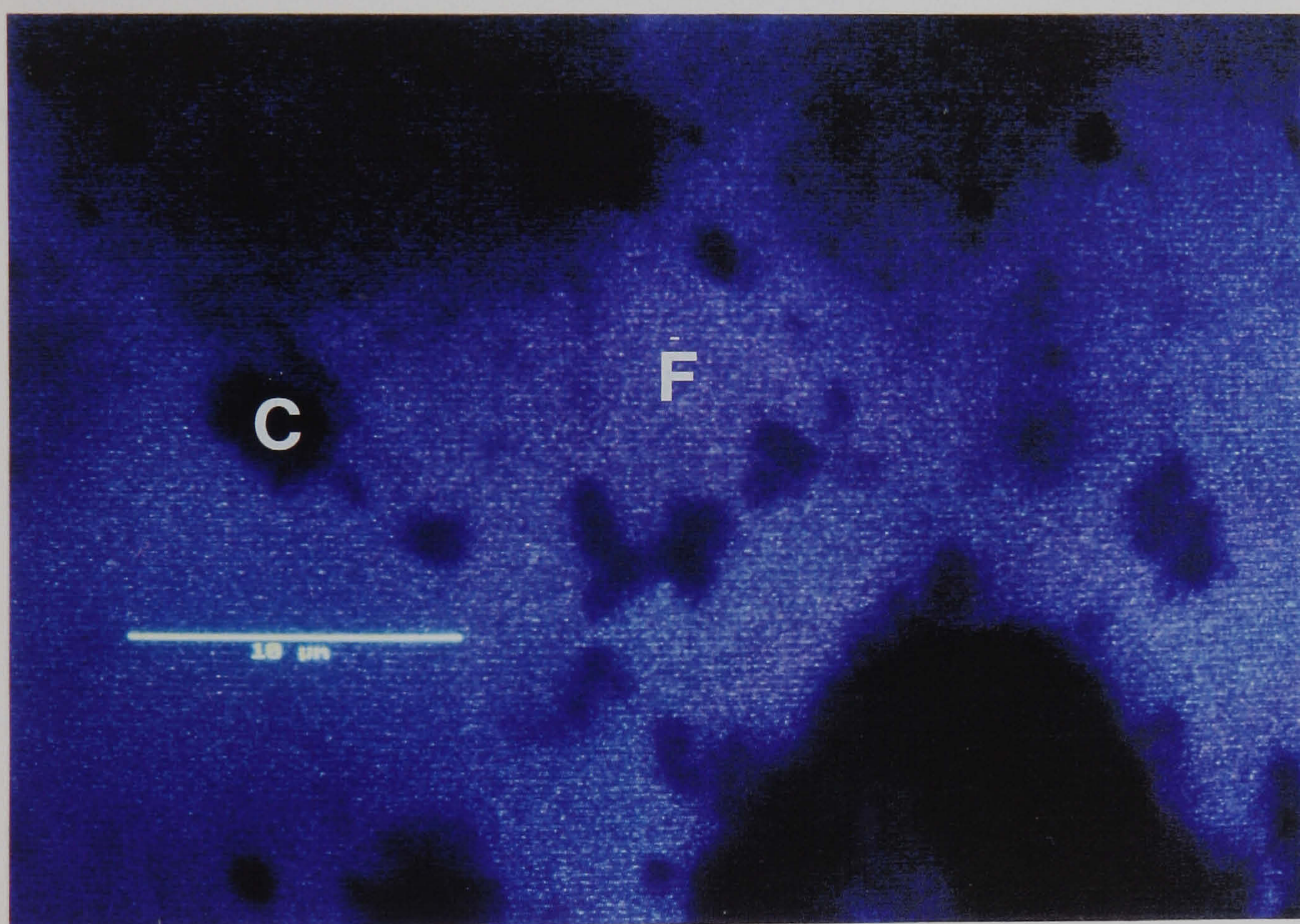


Figure 4.40 Patchy mosaic surface of copper coupons immersed in soft water supplied with particulate matter (marker bar denotes $10\text{ }\mu\text{m}$). C denotes the copper surface and F the fluorescein.

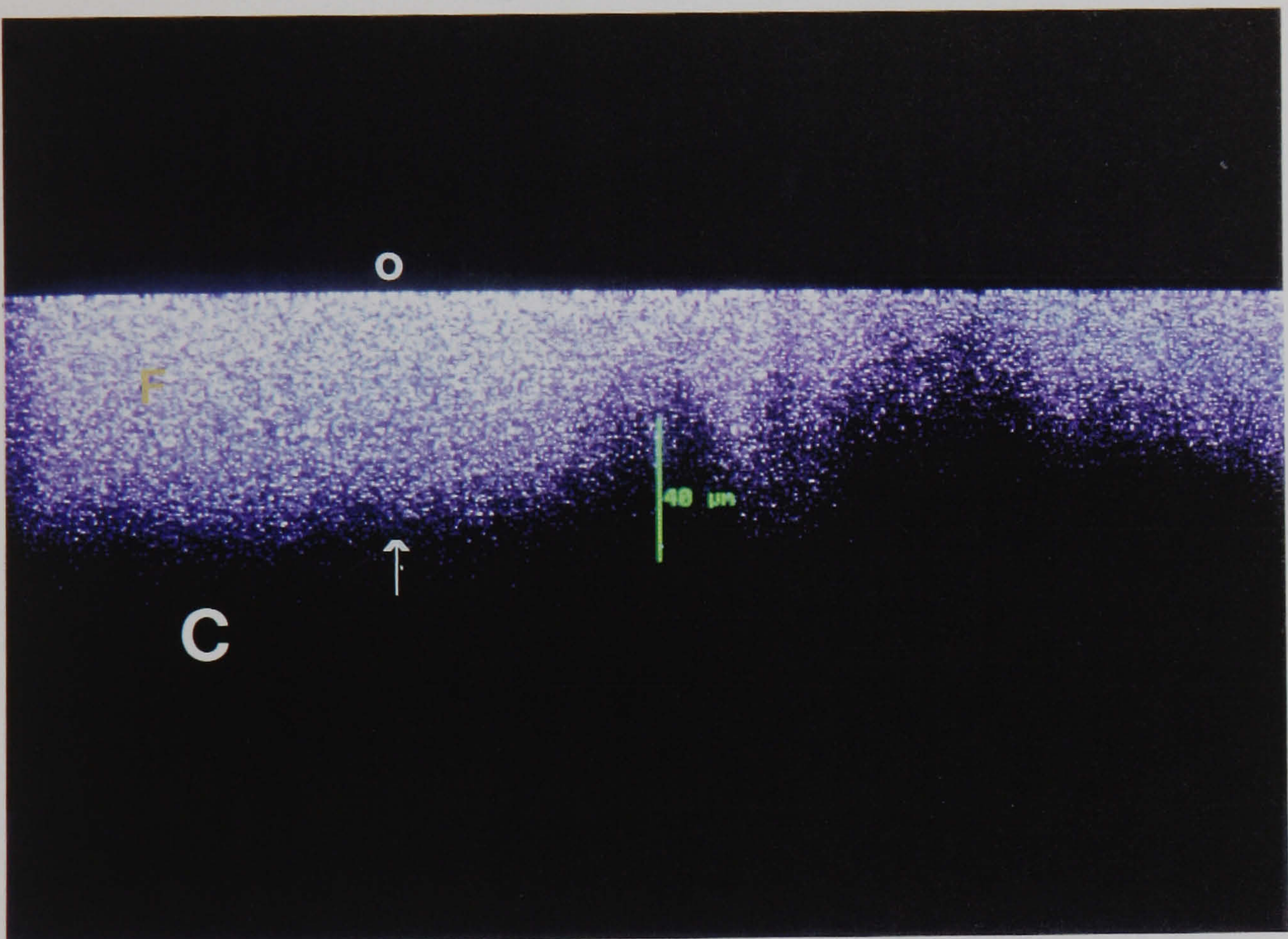


Figure. 4.41 An xz sagittal section through the surface revealed pits of up to $40\text{ }\mu\text{m}$ in the copper coupons immersed in water supplied with particulate matter (marker bar denotes $40\text{ }\mu\text{m}$). F denotes the fluorescein on top of the uneven copper surface (C) with the arrow indicating the inner copper surface and (O) indicating the outer surface).

4.8.4.3 Visualisation of bacteria on the copper surfaces

RITC was utilised to stain the bacteria with fluorescein to study the copper surface on a tile removed from the vessel supplied with particulate matter. Fig. 4.42 demonstrates the bottom of a pit (left hand side image) distributed around which are bacteria (right hand side image).

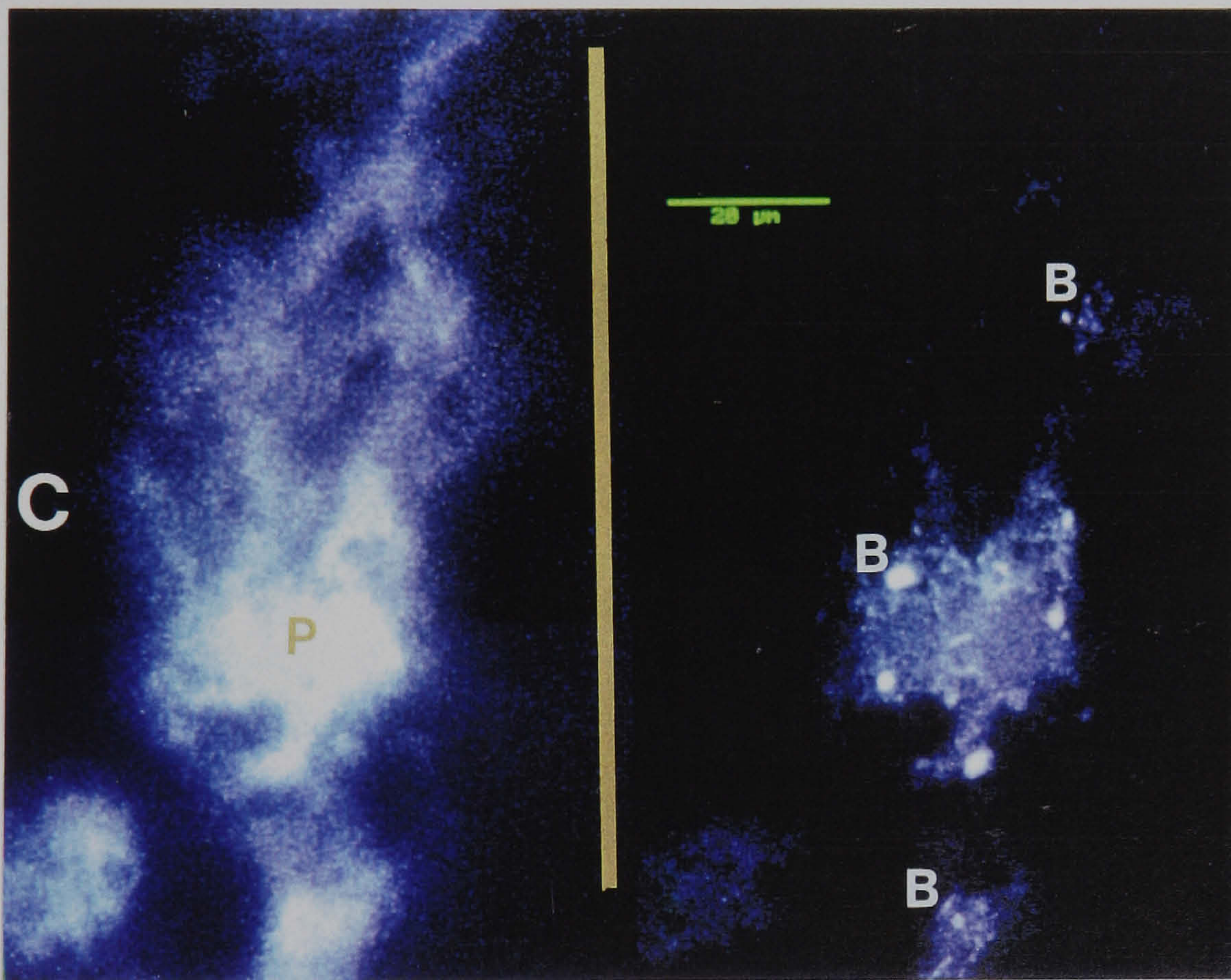


Figure 4.42 Demonstrates the bottom of a pit (left hand side image) distributed around which are bacteria (right hand side image) (marker bar denotes 20 μm). C denotes the copper surface; P denotes the pit and in the right hand image B denotes the position of the bacteria.

Using an xz sagittal section the bacteria were observed to be located on the walls and in the base of the pit in monochrome (Fig. 4.43) and with the facility of colour enhancement (Fig.4.44)

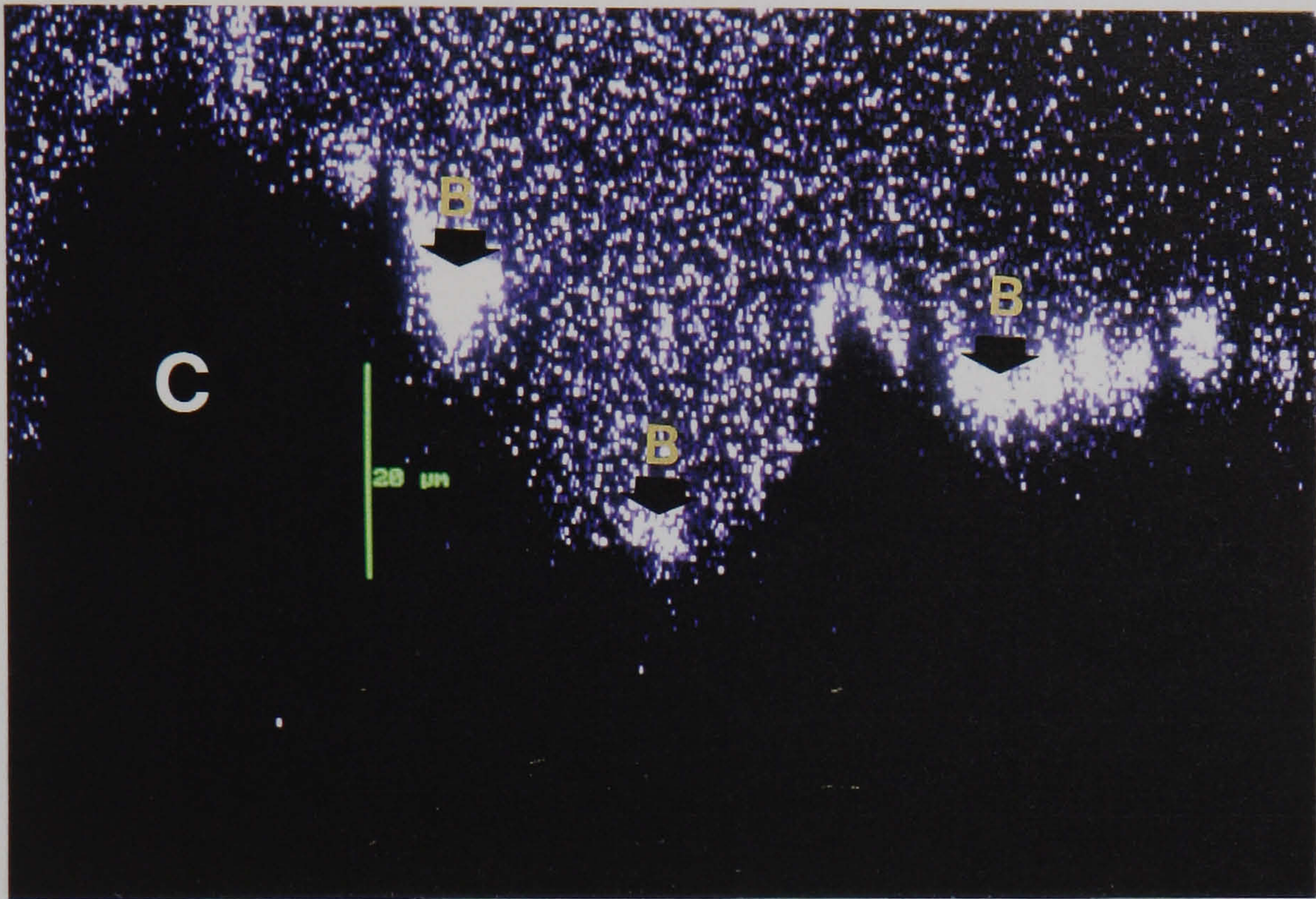


Figure 4.43 Monochrome xz sagittal section of bacteria in pit (marker bar denotes 20 μm). C denotes the copper surface and B denotes the position of the bacteria.

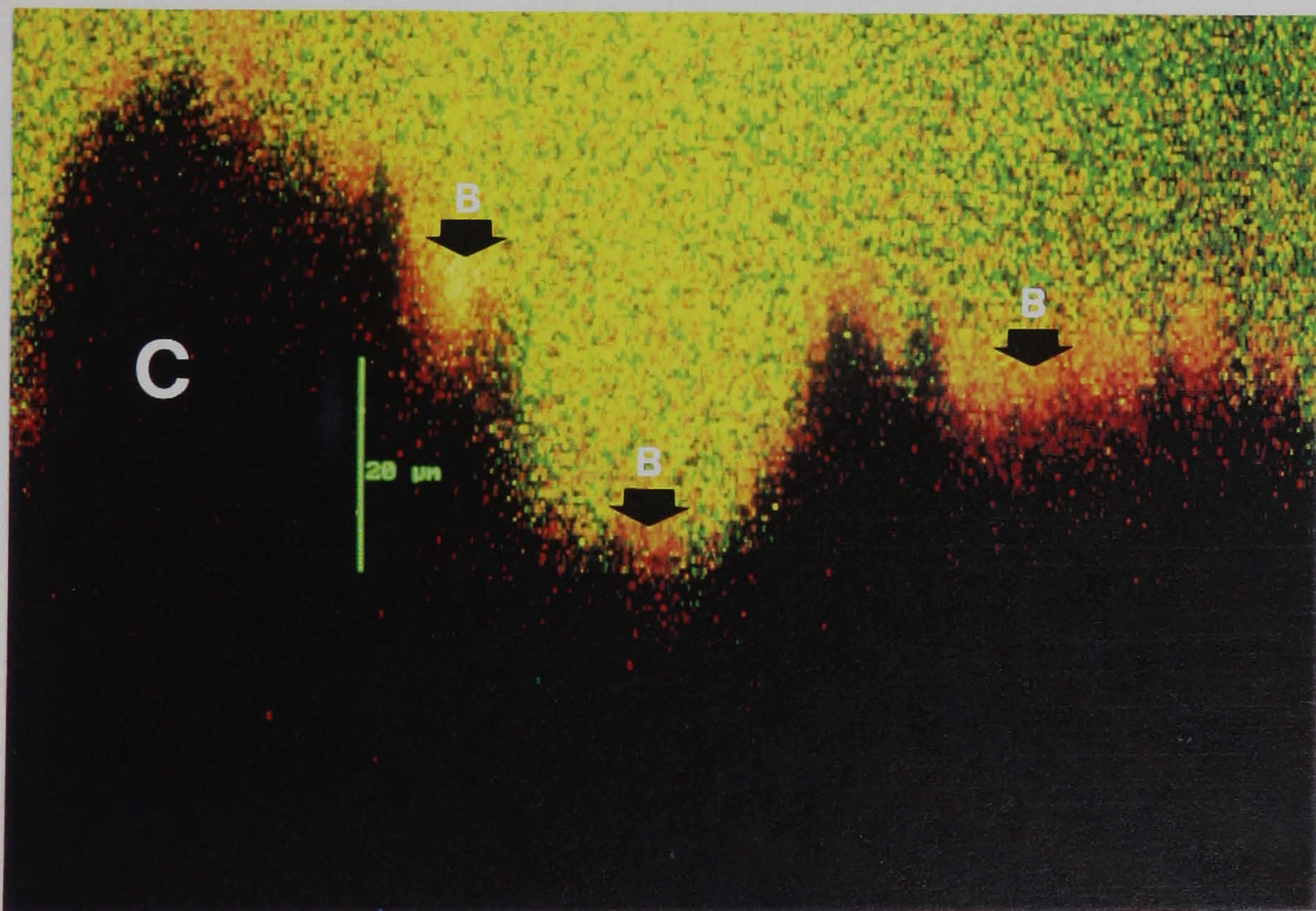


Figure 4.44 Colour enhanced image of xz sagittal section of bacteria in pit (marker bar denotes 20 μm). C denotes the copper surface and B denotes the position of the bacteria.

4.8.4.4 Determination of metabolic activity of the bacteria

The fluorescence spectrum of stain 5-(and-6)-carboxy 2',7'-dichlorofluorescein (CDF) decreases due to acidification and so can be utilised as an indicator of bacterial metabolic activity. Copper surfaces were flooded with CDF then the cells stained with RITC. The surface topology is illustrated in left hand image of Fig. 4.45 while on the right hand image the bacteria can be identified.

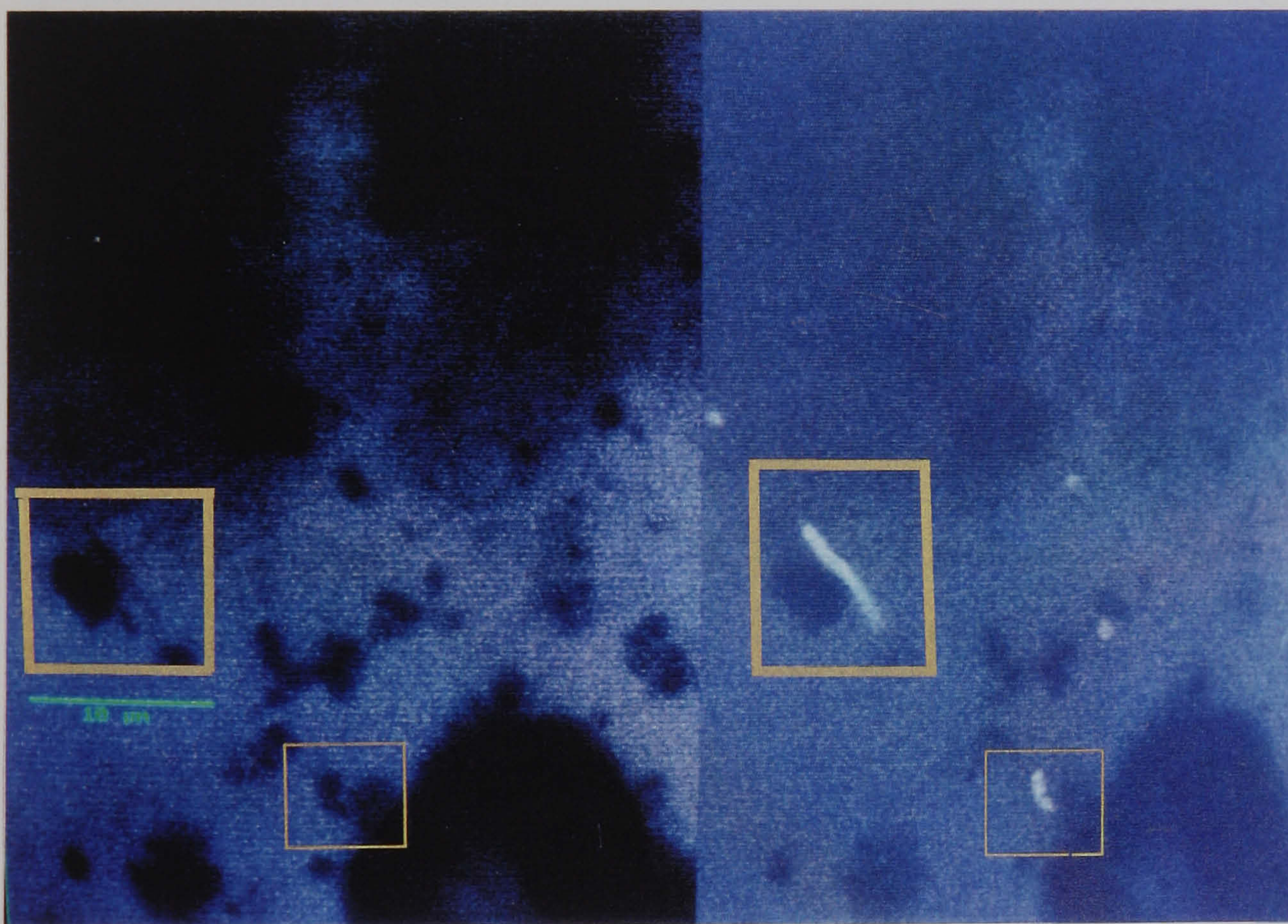


Figure 4.45 Split screen image of surface (left) and bacteria (right) (marker bar denotes 10 μm). The enclosed areas highlight the position of the bacteria and decreased intensity of fluorescence.

When both the images of Fig. 4.46 were merged a number of the areas of acidification can be seen to be associated with the presence of bacterial cells indicative of cell metabolism.

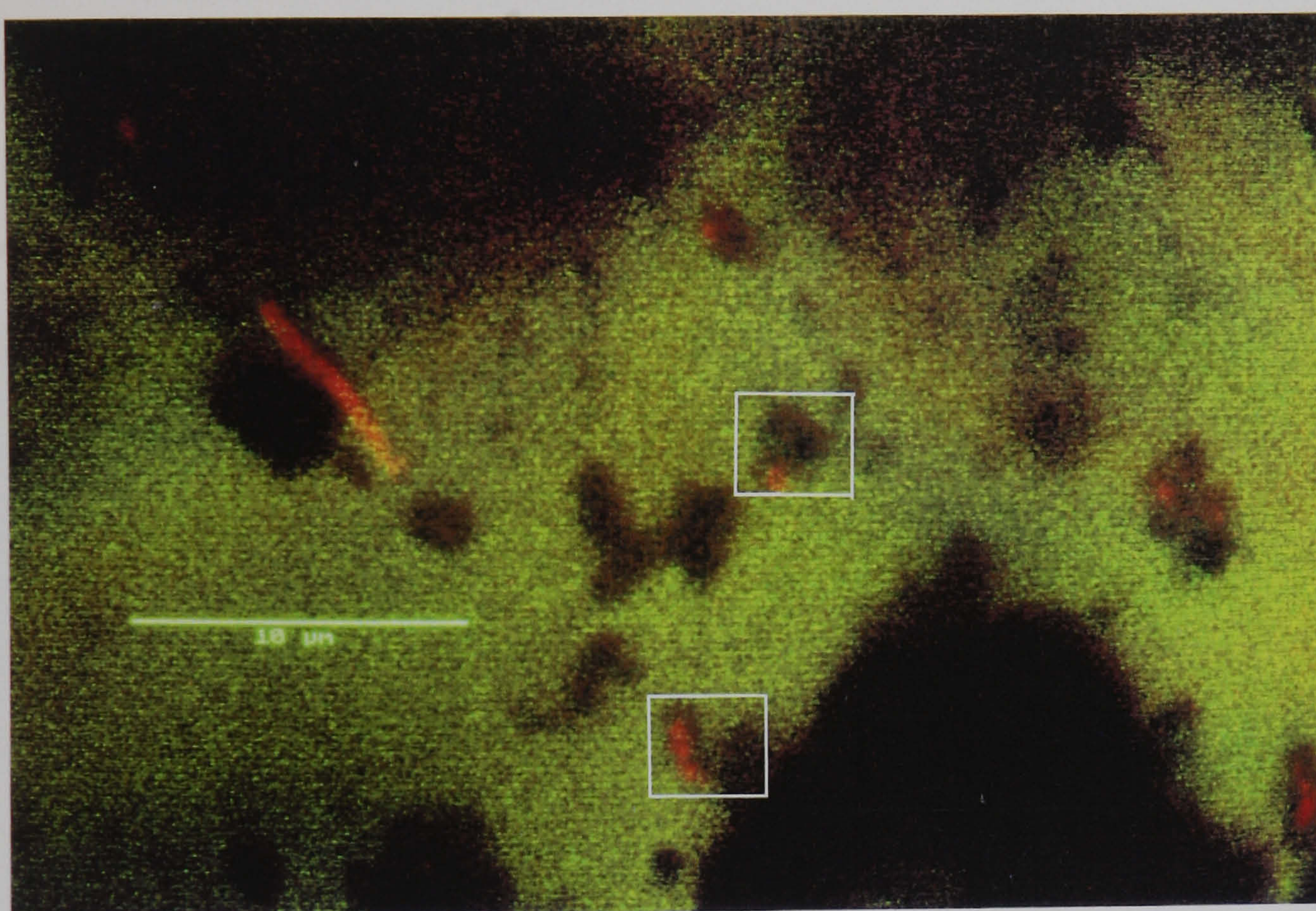


Figure 4.46 Association of bacteria and zones of acidification on the surface of the copper tile (marker bar denotes $10\ \mu\text{m}$). The enclosed areas highlight the position of the bacteria and decreased intensity of fluorescence.

4.8.5 Visualisation of surfaces using epifluorescence, SEM and SCLM.

After twelve months operation sections of the copper tube were removed periodically to be examined for fouling and signs of corrosion using epifluorescence and scanning electron microscopy.

Fig 4.47 shows that fungi were visible on the surface utilising differential interference contrast. However greater discrimination (Fig. 4.48) was obtained after staining the nucleic acids of the cells using acridine orange. Focusing further down into the specimen (Fig. 4.49), individual bacteria could be discriminated on the surface

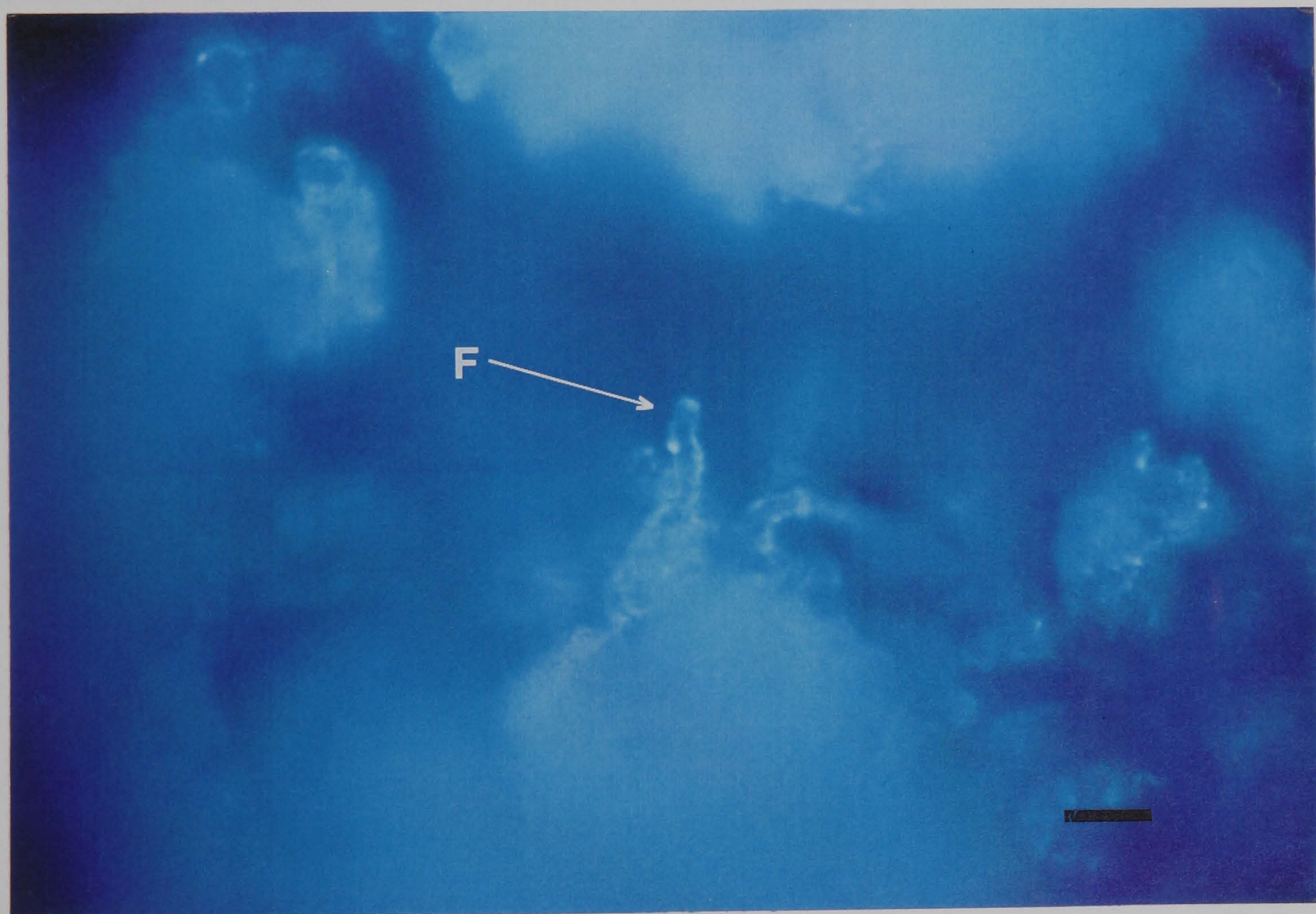


Figure 4.47 Discrimination of fungi on the copper tube surface using differential interference contrast, x 1000 (marker bar denotes $10\mu\text{m}$). F denotes the position of the fungi.

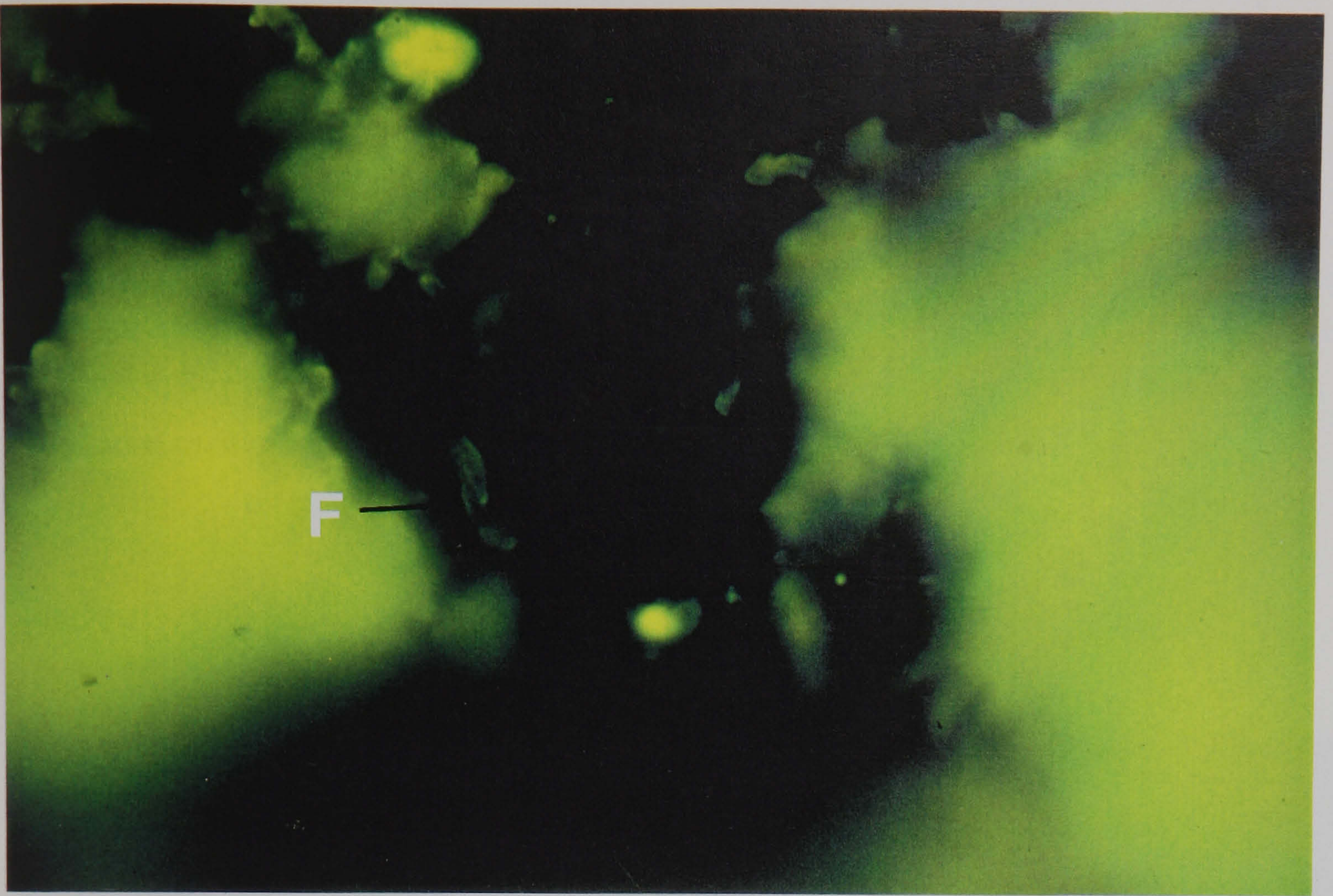


Figure 4.48 Greater differentiation of fungi was obtained after staining the nucleic acids of the cells using acridine orange, x 1000 (marker bar denotes $10\mu\text{m}$). F denotes the position of the fungi.

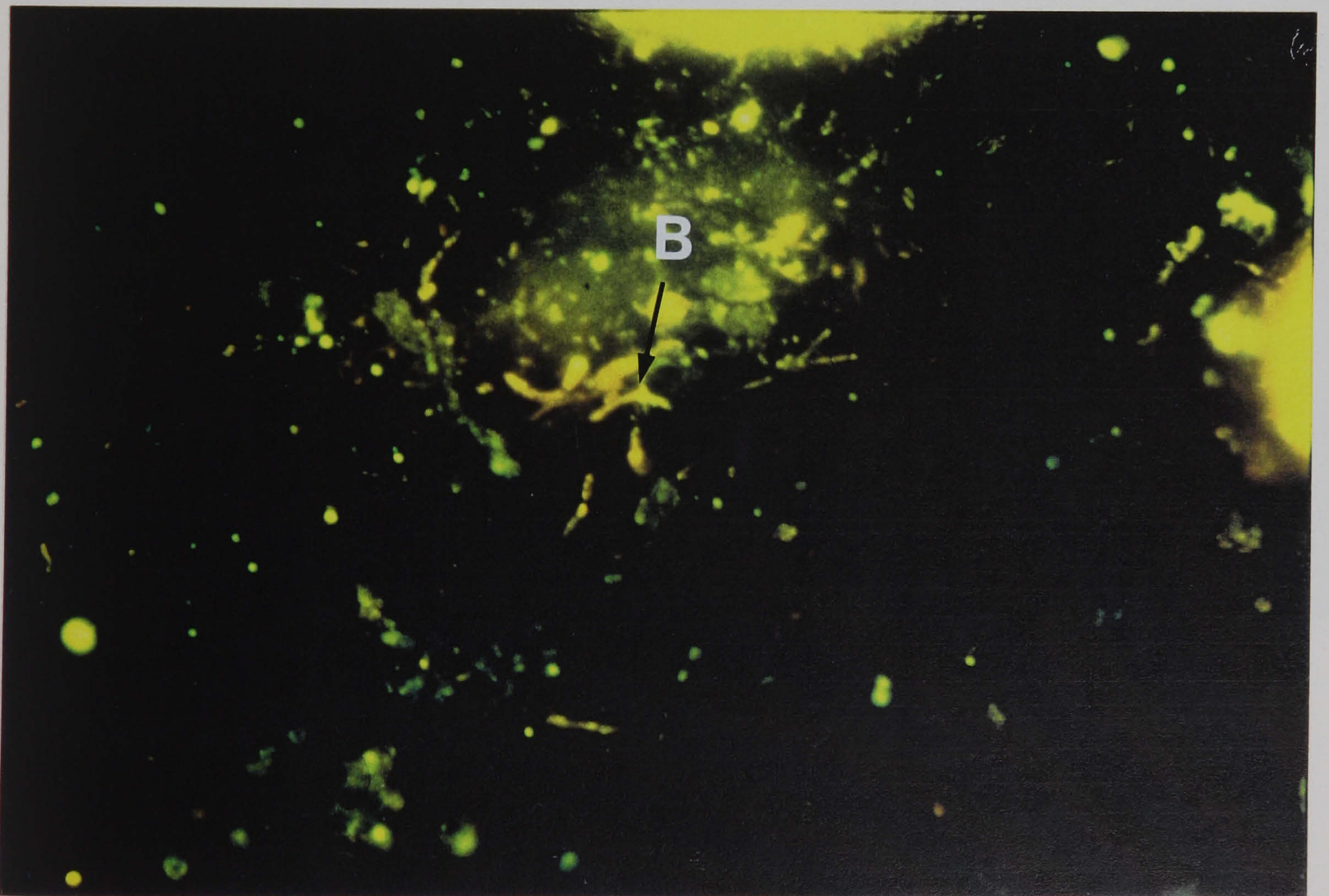


Figure. 4.49 Individual bacteria could also be identified on the copper tube surface, x 1000 (marker bar denotes $10\mu\text{m}$). B denotes the position of the bacteria.

Other copper specimens from the copper tube were visualised using a scanning electron microscope equipped with a cryogenic stage enabling the specimen to be frozen using liquid nitrogen. The specimen was viewed directly but no discrimination was available of the sample and so it was then sputter coated with gold before visualisation. In Fig. 4.50 (x 1862) a number of bacteria can be visualised entwined in a threaded matrix which covers the surface in a matrix.

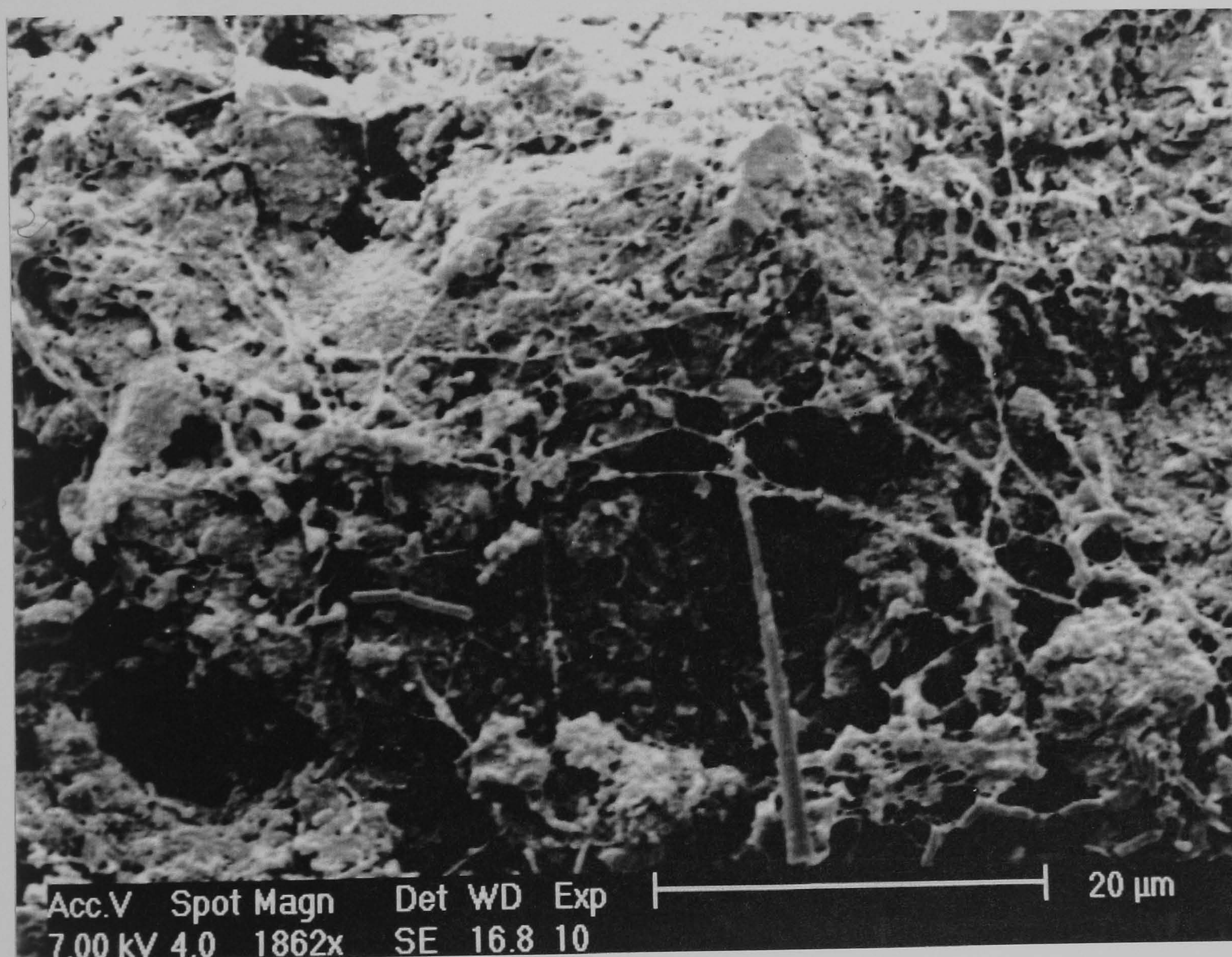


Figure 4.50 A number of bacteria can be visualised entwined and connected in a hydrated network, x 1862 (marker bar denotes 5 μm)).

When the surface of the copper tube specimens were viewed using the ESEM (Fig. 4.51) the bacteria were observed to be covered in a slime and were difficult to identify until the magnification was increased to 8500 times.

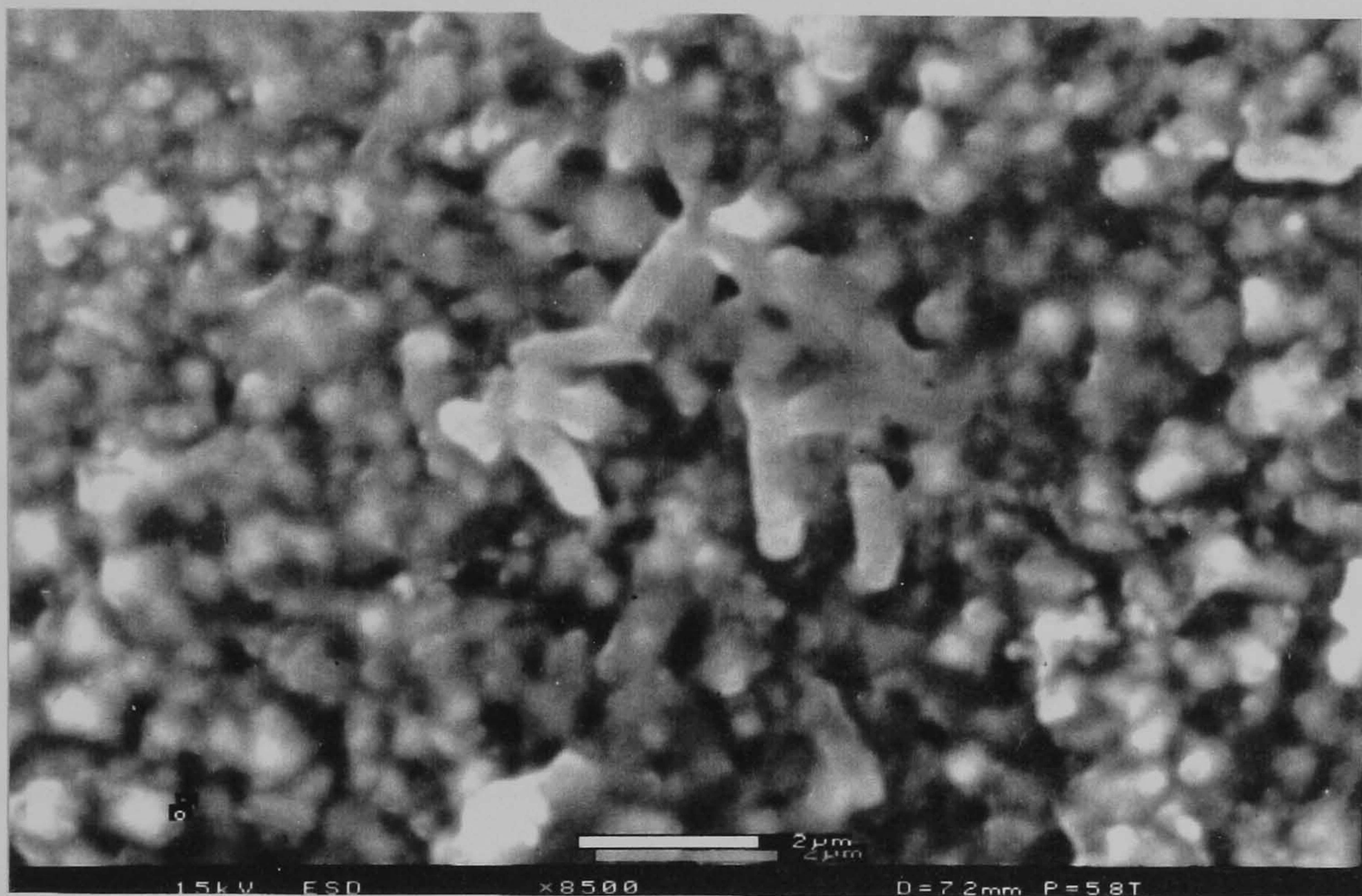


Figure 4.51 Utilising the ESEM the bacteria were observed to be enclosed in a hydrated slime (marker bar denotes 2 μm).

4.9 Discussion

The remit of the laboratory model was to simulate a domestic water system of an institutional building and to investigate the parameters under which colonisation of copper tube was occurring. As described in the material and methods (section 2.5, page 51) the medium used to grow the mixed microbial consortia was filter sterilised tap water with no added carbon sources. The tap water was obtained from the site where copper tube corrosion was occurring. Preparation of the water medium was based on a study by Colbourne *et al.* (1988b) whose method of filtration was used to obtain a sterile, chemically unaltered, natural water medium for use in continuous culture models. The culture vessels used in the present study were composed of inert materials such as glass, titanium and silicone to prevent ingress of exogenous nutrients or metals that may otherwise have altered the water chemistry. Two continuous culture vessels were linked in-series to simulate a water system. The first vessel was used to grow the inoculum under standard and stable conditions to simulate a water tank or calorifier that would produce an inoculum to seed the system i.e. the second vessel, down stream. According to the dynamics of continuous culture, effluent from this first vessel would be composed of starved or spent cells and media. Therefore the second vessel also received supplemental sterile culture medium to revitalise the spent cells flowing in from the first vessel. Biofouling of copper plumbing tube sections suspended in the second vessel was then examined under different conditions to simulate a laminar flow water system being constantly challenged by microorganisms. The advantage of using two vessels in series was that parameters could be changed to study the effect on the planktonic and biofilm phase in the second vessel without affecting the constant inoculum coming from the first vessel.

4.9.1 Colonisation of Copper and glass surfaces

Section 3.4 (page 108) discussed biofilms that were associated with copper tube corrosion at the Victoria Infirmary and Inverclyde Hospitals where the hot water temperatures were between 40-46°C. However in the control sites, Glasgow Royal Maternity, Stratheden hospital and Eastern General Hospital where the water was found to be approximately 55°C, pepper pot pitting corrosion of copper tube was not considered to be a problem. Biofouling was studied in the laboratory model by investigating the ability of a mixed microbial consortium to develop on a substratum in tap water from the Victoria Infirmary between 40-60°C. It was an important factor of this study that the laboratory model was being utilised to simulate a domestic water system and so no other exogenous carbon source was added to the water. Thus, the sole nutrient source for the microbial inocula was the filter sterilised tap water as occurred in the water circuit.

Initially, biofilms were developed in the laboratory model on copper and glass surfaces between 40-55°C. The biofilm coupons had previously been cleaned by degreasing in alcohol followed by autoclaving. However, even though the substrata were subjected to a cleaning procedure they would be instantaneously modified after exposure to the water. This would result from the adsorption of an organic film onto the surface and may have influenced bacterial attachment (McEldowney and Fletcher, 1986; Fazio, *et al.* 1982). Wangersky (1976) demonstrated that such organic layers can consist of glycoproteins, proteoglycans as well as humic residues. Considering that the filter sterilised soft tap water was from an upland catchment area then its typical brown colour (particularly evident by the non-dissolved portion retained after filtering-results not shown) was most probably due to the decomposition of peat, resulting in mainly humic and fulvic acids.

Colonisation rapidly occurred on coupons immersed in the laboratory model within 24 hours between 40-55°C and the type of biofilm observed was characterised by microcolonies dispersed over the surface. A similar non-uniform attachment was reported by Sly *et al.* (1988) who investigated the colonisation of glass over 28 days with biofilms reaching steady state at 7 days. From the results of Banks and Bryers (1992) microcolony formation may not only be a result of growth from the individual bacteria that initially attached to the surface. In their study they presented results where cells of *P. putida* and a *Hyphomicrobium* spp. deposited onto cells of their own species at a much greater rate than to clean glass surfaces. A similar phenomena was also reported by Pringle and Fletcher (1983). Ellwood *et al.* (1982) demonstrated that surface association increased growth rate and Dawson *et al.* (1981) also reported a beneficial effect of increased substrata area on microbial growth but only at low nutrient concentrations. Attachment to surfaces increased the resistance of *Escherichia coli* to cupric ions (Hicks and Rowbury, 1988) thus demonstrating another mechanism by which surface attachment and growth increased bacterial survival.

Bacteria were not initially recovered from nor visualised on surfaces immersed at 60°C in the culture vessel. However, the number of bacteria recovered from the planktonic phase was only decreased by 13 % after an increase from 55°C to 60°C. This would indicate that although bacteria were still growing they had appeared to have lost the ability to attach to either glass or copper surfaces to form a biofilm perhaps due to a physiological change. Maximum numbers were recovered from both glass and copper surfaces at 55°C. Following exposure to 60°C, the culture temperature was increased to 80°C before being returned to 40°C. Upon repeating the temperature profile of 40-60°C (section 4.4, page 149) a greater number of bacteria were recovered from biofilms, representing a 2.0 Log₁₀ cfu cm⁻² difference, in comparison to those obtained previously (section 4.3). Maximum numbers were again recovered from surfaces that had been immersed into the model at 55°C in

comparison to 40-50°C. These results are in agreement with those in section 4.3, suggesting a preference for growth at that higher temperature. Biofilm development was still controlled at temperatures above 55°C representing a 3.0-fold log₁₀ decrease at 60°C.

The importance of temperature in this study is two fold. Firstly, pepper-pot pitting corrosion of copper tube occurred in hospitals where the hot water system was maintained between 40-46°C, but in control hospitals with hot water temperatures greater than 50°C this form of corrosion did not occur (Keevil *et al.* 1989). Secondly, temperatures > 50°C are recommended for the control of *L. pneumophila* (Anon, 1990) within water systems. This is of primary importance within hospitals where this water borne pathogen has been found in the water phase and biofilm from fixtures and fittings (Plouffe *et al.* 1983; Colbourne *et al.* 1984) of water maintained <56°C.

Therefore, there does appear to be a correlation with bacteria associated with pepper pot-pitting corrosion and the presence of *L. pneumophila* in domestic hot water systems that are maintained below 56°C. The number of bacteria recovered from biofilms on glass and copper surfaces were similar between 40-55°C. Other researchers (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983) demonstrated increased bacterial attachment to hydrophobic plastics with little or no surface charge e.g. polyethylene. However, they also found decreased numbers of bacteria attached to hydrophilic metals with a positive charge e.g. platinum and less to hydrophilic negatively charged surfaces e.g. glass. In this particular study there was no difference between glass and copper.

4.9.2 Population profiles on copper and glass

Four groups of microorganisms were identified in the biofilms including *Aspergillus fumigatus*, *Pseudomonas* spp., *Methylobacterium* spp. and Gram negative bacteria. At 40°C the four microbial groups were present on both copper and glass surfaces with the Gram negative bacteria dominating. *Pseudomonas* spp., *Methylobacterium* spp. and *A. fumigatus* were transiently present on both surfaces. At 45°C the species diversity decreased as only *A. fumigatus* and the Gram negative bacteria that dominated were present. On biofilms recovered from copper and glass the species diversity increased as the temperature was increased to 50°C. Although all four species were present there was a transient dominance between *Pseudomonas* spp. and the Gram negative bacteria. At 55°C the species diversity decreased again with the *Pseudomonas* spp. being dominated by the Gram negative bacteria.

4.9.3 Comparison of total (SEM) and viable plate counts

Counting viable bacteria using standard plate count methods relies on counting colonies that have grown from single cells. There are many problems with this technique for counting bacteria including, under-estimation of viable bacterial numbers (Peele and Colwell, 1981), agar incompatibility and nutrient shock (Reasoner and Geldrieck, 1985) as well as the time-period required for incubation (Jones and Simon, 1975; Gibbs and Hayes, 1988). However, it still remains the most practised technique for obtaining viable counts of bacteria. Also, viable plate counting is also very useful for recovering bacteria for studies of population profiles as well as culturing bacteria for identification, as was carried out in this study. In addition to the results obtained by standard plate count methods the photo-micrographs from SEM examination were studied to provide an alternative technique for assessing bacterial biofilms.

Scanning electron micrographs of copper coupons removed from the culture vessel demonstrated that bacteria were present at day 21 between 40-55°C, although on some days no bacteria were observed on the coupons e.g. day 1 at 40°C as well as days 1, 7 and 14 at 55°C (section 4.3.2.2, page 131). However, no bacteria were observed on any of the copper coupons extracted from 60°C. This correlates with no viable bacteria being recovered from copper coupons immersed in the vessel at 60°C and as suggested in section 4.81 there may have been a physiological change in the bacteria that prevented them forming biofilms. In the samples where no bacteria were visible by SEM the bacteria may have been buried within the corrosion product on the surface as demonstrated by Blunn, (1987). Alternatively, the biofilm may have been removed during preparation of the samples for SEM. Indeed, Chang and Rittman (1986) reported the loss of biofilm owing to the harsh sample preparation for SEM. Using scanning and transmission electron microscopy Eighmy *et al.* (1983) were able to maintain the morphology of ageing biofilms using the facility of critical point drying prior to transmission electron microscopy. Van Neeren *et al.* (1990) used freeze-drying, freeze-substitution and critical point-drying to study bacteria in polymer beads but also found that shrinkage occurred with all three methods. Richard's and Turner, (1984) also demonstrated the removal of slime and bacteria from pumice during critical point drying, however they were able to minimise this loss using sputter-cryo examination.

4.9.3.1 Visualisation of bacteria on copper surfaces

To enhance the visualisation of bacteria on copper surfaces, Bremmer *et al.* (1992) obtained a smooth layer by polishing copper surfaces with 0.05 μ alumina particles that resulted in increased bacterial counts in comparison to unpolished surfaces. The copper surfaces in this study had not been polished as this would have deviated from the concept of simulating conditions under which the fouling was occurring in the domestic hot water circuit on unpolished copper tube surfaces. The roughness of the

unpolished surface will have provided areas in which the bacteria would have been difficult to observe using SEM. Crevices and milling lines are often colonised first as they are areas of low shear and so would provide suitable sites for initial colonisation as described by Richards and Turner (1984) for pumice stone. Although not shown, individual bacteria were present in the micrographs of coupons extracted from 45°C at days 1, 4 and 7 but at days 14 and 21 bacteria were held in a layer of glutinous like material. Observing bacteria initially as a single bacterium (day 1) then as a microcolony followed by a complex network covering the surface indicates growth from individual bacteria into microcolonies occurring over the copper surface. Banks and Bryers (1992) also demonstrated preferential attachment of bacteria to bacteria rather than bacteria to surfaces that would result in enlarged microcolonies. At 50°C bacteria were not identifiable until days 14 and 21 where the bacteria were interwoven between the mesh like glutinous material on the copper surface. Indeed on copper surfaces bacteria to bacteria attachment may have been preferential considering the toxicity of copper to bacteria (Versteegh *et al.* 1989; Pyle *et al.* 1992)

The bacterial numbers calculated from the micrographs were found to be greater than the viable counts obtained from scraped coupons. In two (at 40°C and 50°C) of the three samples where bacteria could be individually counted on the copper surface there was 1.0 Log₁₀ cm⁻² difference between the total and viable counts. In the third sample at 55°C there was only a 0.1 Log₁₀ cm⁻² difference. Comparing culturable counts against microscopic enumeration, Liebert *et al.* (1983) found that the microscopic counts on formvar coated copper electron microscopic grids differed by 0.2-1.0 Log₁₀ bacterial counts.

4.9.3.2 Visualisation of bacteria on glass surfaces

The number of bacteria attached to glass coupons as visualised by SEM was also enumerated. On glass coupons the bacteria were easily observed. In agreement with the viable numbers, attachment occurred rapidly within 24 hours. In the case of total bacteria observed, the numbers were relatively constant at 40°C from day 1 to day 21 (page 147), whereas, the viable numbers increased from day 1 to day 14, followed by a decrease on day 21. The total bacteria observed was greater than the number obtained by standard plate count technique. At 45°C the total bacterial numbers were also at least $1.0 \text{ Log}_{10} \text{ cfu cm}^{-2}$ greater than the viable numbers recovered. With an increase in the temperature to 50°C both total and viable numbers increased from day 1 to reach a maximum at 50°C on day 7 before decreasing again on day 14. Although the viable numbers then decreased again the total numbers in fact increased.

At 55°C the total numbers increased from day 1 and again from day 7 to day 14. and remained constant on day 14 and 21. However the viable numbers decreased from day 1 but then exhibited a 2-fold increase at day 21 suggesting that the viable number recorded on day 7 could be subject to a large degree of error and may indeed be of a higher value. In tables 4.12 to 4.15 (page 147) the errors for the counts have been tabulated and this demonstrates the large range of errors that result when calculating total numbers in biofilms. The results obtained from the micrographs are based upon the actual numbers obtained from specific areas of the coupons which have been photographed by an independent operator. In doing so the operator has tried to produce a set of micrographs which are representative of the whole area of each coupon. Therefore, the glass coupons immersed in the continuous culture vessel exhibit microcolonies surrounded by sparse areas (page 144). It is by observing the micrographs that a picture of how the biofilm forms is achieved and that the large errors are incorporated. Biofilms in this present study were not homogenous layers of cells, as was also demonstrated by Weber *et al.* (1978), who studied the physical and

structural characteristics of microorganisms on activated charcoal in water and waste water applications. Whereas, Wimpenny (1988) designed a laboratory model to develop biofilms of 300 μ in depth, the results presented here demonstrate that the biofilm obtained in the continuous flowing model are heterogeneous in their nature resembling a mosaic and are not 300 μ deep. However, Wimpenny (1988) operated a model to investigate biofilm physiology at different depths for which the thick and constantly reproducible biofilm generator was immensely useful. Total counts were obtained from every glass tile immersed in the continuous culture vessel (tables 4.12 - 4.15) whereas bacteria were difficult to observe on copper coupons (table 4.4 - 4.7).

4.9.4 Effect of temperature on the planktonic population

Vessel one, where conditions were constant, was used to establish the inoculum. The temperature was maintained at 30°C as this was the lowest temperature at which surveyed hot water systems had been found to operate (section 3.3, page 77). This first vessel was maintained to supply a constant inoculum, an average of 4.9×10^5 cfu ml⁻¹ (5.6 log₁₀) over the first seven months, to the second vessel where biofilm generation was carried out. It was during this seven months that the temperature of the second vessel was changed from 40°C to 70°C. Fluctuations in the number of bacteria from the planktonic phase between 40-60°C do not appear to be temperature dependent as bacterial numbers are similar in both vessels. Only when the temperature was changed to 65°C was a 1.0 Log₁₀ cfu ml⁻¹ decrease in planktonic bacterial numbers observed. The number of bacteria recovered from biofilms are scraped from 1 cm² and cannot be directly compared to the number recovered from 1.0 ml of the planktonic phase. Bacterial biofilm numbers were similar at 40°C and 50°C, at 2.5 Log₁₀ cfu cm⁻² and were higher at 45°C at approximately 3.7 Log₁₀ cfu cm⁻². Indeed, the greatest number of bacteria were recovered from copper coupons at 55°C with no biofilm bacteria recovered at 60°C. This is in contrast to 4.5

$\times 10^5$ cfu ml⁻¹ of viable bacteria in the planktonic phase. Therefore, the bacteria are viable but are unable to form biofilms. Bacteria extracted from the planktonic phase were viable as they were recovered on agar plates after incubation at 30°C, but were the bacteria actually growing in that second vessel? The resident time spent in the second vessel may not have been long enough to be bactericidal, in which case 60°C may only have been bacterio-static. As there was no growth on plates incubated at 60°C (results not shown) the population was not composed of thermophiles or alternatively this agar was not sufficient for the recovery of stressed or thermally injured bacterial.

Spent cells and media, that is, the effluent from chemostat one, $D = 0.05$, was pumped into chemostat two which also received 75 ml per h filtered sterile soft water media resulting in a total flow rate of 100 ml per h or $D = 0.2$. Considering that chemostat two receives 25 ml per h from chemostat one then if growth does not occur the number of bacteria flowing into chemostat two will be the same as that leaving chemostat one. As chemostat two receives another 75 ml media the flow rate within it will be 4 times faster than that in chemostat one and so for every ml extracted from chemostat two there should only be 25 % the number of bacteria from chemostat one. However, the number of bacteria recovered from the planktonic phase between 40-60°C is similar between the two vessels. Therefore, growth was occurring within the planktonic phase at 60°C even though biofilms were not initially generated. Bacterial numbers in vessel two were only reduced to 25 % of that retrieved from vessel one (30°C) when the temperature was increased to 65°C. This indicates that in a continually flowing system of two vessels linked in-series that 65°C was also only bacterio-static. Indeed, when the temperature was increased to 70°C <10 % of the viable bacteria flowing in from vessel one were recovered from vessel two with a further decrease in the planktonic numbers at 80°C. Therefore, temperatures of 65°C were bacterio-static while temperatures above this were bactericidal. Bacterial numbers only recovered when the temperature was returned to 40°C. However, the

results show the importance of a recirculation system for the dissemination and growth of bacteria that are able to remain viable even though conditions are unfavourable.

4.9.4.1 Percentage profiles characteristics

Planktonic populations were initially dominated by the Gram negative bacteria at 40°C similar to those from copper and glass surfaces. While the Gram negative bacteria continued to dominate in the biofilms there was a change in the planktonic phase with Gram negative bacteria succeeding from 45-60°C. At 55°C there was a decrease in the species diversity as *Methylobacterium* spp. were not recovered from the biofilm but were still present in the planktonic phase. The above results, where different bacteria dominate under different conditions, illustrates the value of approaching a study of a mixed microbial consortia from a holistic point of view. In other studies where pure culture strains have been used to study colonisation then no data would have been obtained on the importance of other bacterial strains on the process (Wright *et al.* 1991). Information obtained from population profile studies are important if biofilm control using chemical means is to be considered. Historically, biocides have been chosen due to the number and type of bacteria recovered from the water phase (Costerton and Geesey, 1979). However, results from this present study demonstrate that different bacterial species exist under different conditions and so one biocide chosen to act against a certain planktonic population may not be as efficacious against the biofilm that may contain a different species of bacteria.

During the repetition of the temperature profile the ratios of the bacterial types changed as the Gram negative bacteria predominated and the *Methylobacterium* spp. represented a greater proportion of the biofilm. Indeed, at 55°C and 60°C the *Pseudomonas* spp. that dominated in the first temperature profiles were also succeeded by the *Methylobacterium* spp. This switch in the dominance of planktonic

bacteria may have contributed to the presence of bacteria on the surfaces when the coupons were removed from the culture at 60°C in the repeat experiments (section 4.4, page 149).

4.9.5 Control of Fouling

Water treatment plants and distribution networks function to provide potable water to existing legal standards and to ensure that there is a sufficient quantity at every point within the supply area (Block, 1992). Existing legal standards are set to ensure that water leaving treatment plants does not contain pathogens even though only less than 4 % of distributed water is used for human consumption (Block, 1992). Most pipe surfaces are colonised by organisms such as diatom, algae, filamentous and rod shaped bacteria (Allen, *et al.* 1980) and therefore water leaving water treatment plants and entering the domestic water supply should be free from pathogens but may not be sterile. In the majority of hot and cold water circuits this may not lead to any problems, however, attachment of organisms to pipework will cause an increase in fluid frictional resistance resulting in increased power consumption for pumped systems and also reduced capacity (Characklis, 1981). Methods to control the presence of bacteria in water distribution systems include chemical mechanisms such as increasing the free chlorine residual concentration (LeChevallier, 1987) or physical mechanisms including flow driven nylon brushes (Nickels, *et al.* 1981) to remove biofilm (pigging).

As biofilms had been implicated in the copper corrosion process, mechanisms of retarding biofouling and maintaining a biofilm free surface in the copper pipe network required investigation. Using biocides or disinfectants was not considered by the relevant authorities and so thermal pasteurisation was suggested to control biofouling in the hot water system. However, for water circuits that were already fouled the

biofilm would have to be removed from the surfaces. The possibility of using citric acid to remove biofilm from copper tubes surfaces in the County Hospital, Hellersen, (Germany) was discussed (Fischer *et al.* 1991) as it was the only promising reagent permitted by German food law. In response to a plan to use citric acid in site work the method was also evaluated in the laboratory along with the use of sulphamic acid for comparison as it was indicated that this may also be used to remove biofilm.

4.9.5.1 Effect of pasteurisation on biofilms

Thermal pasteurisation, for the control of *Legionella pneumophila*, by raising the temperature of the whole hot water system to 60°C for at least one hour with the temperature at the outlets reaching 60°C has been recommended in the Health and Safety report (Anon, 1990). Calorifiers should routinely be maintained at 60°C with at least 50°C attainable at the taps. From the initial laboratory results biofilms were generated at 55°C but were controlled at 60°C. Therefore 60°C was used to for pasteurisation. Biofilm was established at 45°C (Fig. 3.4a) and after only 15 minutes exposure to 60°C there was a 99 % reduction in the recovery of biofilm bacteria. The Gram negative bacteria dominated before and after pasteurisation. Dewailly and Jolly, (1991) reported that the dissemination of *L. pneumophila* occurred only when the outlet water temperature was below 56°C indicating the thermal pasteurisation may have beneficial results in the control of *L. pneumophila* and may also be preventing fouling.

Biofilms were also generated on copper and glass control surfaces with the temperature being changed sequentially from 50°C, 55°C, 60°C then to 45°C over a 50 day biofilm development period. After 1 h exposure to 60°C the number of viable bacteria recovered from copper surfaces had decreased by 59 % and by 92 % after 2h. The decrease in the viability of bacteria from glass surfaces was only 21 % and 30% after 1 and 2 h pasteurisation at 60°C respectively. Such results may indicate a

synergistic effect between thermal pasteurisation and the presence of copper surfaces on bacterial viability in comparison to the results obtained on glass. Gram negative bacteria dominated on both surfaces before and after pasteurisation (Fig. 4.5).

Copper toxicity (Bartlett *et al.* 1974) has been recorded for over 100 years and may have contributed to the 92 % decrease in bacterial recovery from copper coupons pasteurised at 60°C in comparison to only a decrease of 21 % from glass coupons. Versteegh *et al.* (1989) were concerned about the recovery of *Aeromonas* spp. coliforms and faecal streptococci from water samples containing copper and recommended the addition of disodium-ethylene-diamino-tetraacetate (Na₂EDTA) to neutralise the toxic effect of copper. An investigation of copper toxicity on the growth of coliform bacteria indicated that cells injured by the presence of copper may have an impaired respiratory system. The binding of copper to the thiol groups of bacterial respiratory enzymes may have inhibited enzyme function (Domek, *et al.* 1984). However copper is an essential metal required for several bacterial enzymes, many of which are involved in biological electron transfer of oxygen utilisation (Petola *et al.* 1993). A number of functions are recognised by which bacteria are able to reduce the toxic effects of copper. Bitton and Friehofer, (1978) compared a polysaccharide producer, *Klebsiella aerogenes* against a non-polysaccharide producing mutant and found that when exposed to copper, the polysaccharide producing strain exhibited increased survival rates. Protection afforded by polysaccharides that chelate copper has also been shown to protect marine bacteria from the toxic effects of copper (Corpe, 1975). West *et al.* (1990) demonstrated that bacteria colonising glass in the presence of copper pipe produced significant quantities of exopolymer as a defence mechanism against leaching copper ions. Marszalek *et al.* (1979) also detected bacteria that secreted extracellular mucoid as a protection mechanism in the presence of toxic metallic ions. Another mechanism of resistance has been found in cells of *Pseudomonas syringae* that were recovered from plants to which antimicrobial copper compounds are applied for plant disease control. These

copper resistant strains were found to be carrying DNA that encoded a periplasmic copper binding protein. Therefore this sequestration of copper outside the cytoplasm has been proposed to act as another protection mechanism. Such defensive mechanisms by bacteria against the toxic ions of copper would no doubt be of benefit in the fouling of copper tubes in water systems maintained between 35-50°C as was found in the sites experiencing corrosion in the site survey.

4.9.5.1 Treatment of established biofilms with acids

The county hospital in Hellersen (Germany) was built in 1982 and opened in 1986 and the first copper tube failures due to pitting corrosion occurred within a few months (Fischer, *et al*, 1992). As well as replacing 30 % of the pitted copper pipe they also treated sections with 10 % (v/v) citric acid in an effort to stop the corrosion.

Citric acid was used in the County Hospital in Hellersen as a mechanism of preventing further corrosion by removing the biofilm and corrosion products to leave a clean copper surface. However, after the citric acid rinsing, the formation of corrosion products and biofilms in the treated hospital copper tube sections started to occur again (Fischer *et al*. 1992b). Using citric acid in the present laboratory model it was clear that bacterial biofilms recovered to similar numbers within 14 days of treated copper coupons being replaced into the culture vessel. In comparison recolonisation of copper coupons treated with sulphamic acid (5 %) only represented 6 % of that obtained after 14 days maturation. Two possible reasons may explain why the citric acid treated coupons were recolonised so quickly. Firstly, it was considered that all of the citric acid may not have been rinsed from the coupons before re-immersion into the culture vessel and that some of the residue may have been converted to citrate (at neutral pH) which may be a nutrient source for certain types of bacteria e.g. *Klebsiella*. One group of the Gram negative bacteria have been found to be citrate utilisers but there was no positive identification. Alternatively, Gadd and White

(1989) state that citric acid is an efficient chelating agent providing an initial pellicle layer on the surface. Therefore, if residual citric acid was present on the coupons which were re-immersed into the culture then it may be possible that the citric acid would have either chelated copper ions and/or the conversion to citrate would have provided a nutrient source for certain bacteria which would proliferate on the surface.

Considering the above results, citric acid does not appear to have prevented the re-occurrence of biofouling in the laboratory model or for that matter in the hospitals where copper tube failures have reappeared (Fischer *et al.* 1992b). The laboratory results with sulphamic acid indicate that it could have reduced rapid recolonisation and may have been a more appropriate cleanser than citric acid.

4.9.6 Altering the chemistry of the soft water

The total hardness of the soft water in central Scotland was less than 30 ppm as was the total alkalinity indicating a very soft and weakly buffered water. Due to this the potential for deposition of protective carbonate films is minimal and excessive metal corrosion is a common problem in such type of waters (Reiber, 1987). An alternative mechanism of protecting copper surfaces from corrosion is the provision of such a protective layer on the surface.

By increasing the hardness and alkalinity of the water by the addition of calcium carbonate it was intended to provide a homogenous oxidised protective coating on the copper surfaces. Reiber, (1987) successfully demonstrated an approximate 40 % reduction in the corrosion of copper plumbing material by increasing the concentration of CaCO_3 three-fold. Increasing the carbonate concentration within the water supply to a unit of a hospital in central Scotland to decrease the corrosion rate was suggested. However, although this mechanism of protection was to decrease or

prevent corrosion of the copper surfaces its effect upon the bacteriology of the source water was not known. Therefore, the laboratory model was used to ascertain if altering the water hardness by the addition of calcium carbonate would have any effect on the formation of biofilms.

A comparison of colonisation of copper coupons in the absence and presence of additional calcium carbonate revealed that greater numbers of viable biofilm bacteria were recovered during periods of higher concentrations of calcium carbonate. As well as providing a protective layer on the surface of the copper the carbonate may also be protecting the bacteria from bacterio-static or bactericidal properties of the copper. In an investigation of pitting corrosion in a distribution pipeline Tuovinen *et al.* (1980) found a large number of tubercles on cast iron pipes were associated with bacteria and pitting corrosion. In one particular site where reduced pitting corrosion was observed it was indicated that less tuberculation perhaps arose due to the protection of a higher concentration of CaCO_3 in comparison to the other sites. Lining pipes with a layer of CaCO_3 was then suggested as a mechanism to prevent the corrosion process. As such results by Tuovinen *et al.* (1980) appear to contradict those obtained in this present study.

4.9.7 Visualisation of copper and biofilm formation

4.9.7.1 Environmental SEM

The idea of using an ESEM is that the morphology of the biofilms that are examined should not collapse as the structure is maintained under pressure when the water is sublimed off. Maintaining the morphology of the specimen is an important feature of any visualisation technique chosen to study the topography of biofilms. An important criterion with this method is that it is the surface, inclusive of biofilm which is being studied. If one wished to look at the copper surface direct then the biofilms would

have to be removed. Therefore artefacts may then be introduced into the sample and as such one has to identify the advantages which this method can offer and also recognise the limitations of the technique. Those limitation are readily understood when other techniques are used which can simultaneously study the bacteria and surface topography e.g. SCLM, however this technique is unable to reproduce the type of morphological information obtained from an SEM.

Image analysis was used to compare surface morphology of the copper coupons extracted from the laboratory model. On copper coupons supplied with particulate matter, 600 countable objects were identified, with only 147 countable objects identified on the coupons from the vessel not supplied with particulate matter. When operating the image-analysis, identifiable objects i.e. the bacteria have to be labelled. This is carried out by a measure of intensity as the bacteria are intensified in comparison to the background (Fig 4.33-4.37). However, in the case of the tile from the vessel with no particulate matter the tile surface is intensified because the bacteria are present in a crevice of the surface and more water had to be sublimed off (Fig. 4.34). Danilatos *et al.* (1984), was able to demonstrate that spores of *Bacillus apiarius* retained their shape whether wet or dry when visualised with the ESEM and so the morphology of the bacteria on the copper surface would expect to be true.

Due to sublimation of the water, areas of the surface have become intensified resulting in the differential between the two surfaces not being greater. Translating these identifiable objects into areas then in comparison only 24 % of the tile not supplied with particulate matter was covered with biofilm material. The copper pipe which also had water containing particulate matter flowing through it had less copper surface visible (Fig. 4.32).

These results indicate that particulate matter may have a role in encouraging the growth and density of biofilm on surfaces. 5 μm spaces or channels were also demonstrated in the infrastructure of the biofilm and may allow the passage of water

which would replenish the biofilm with fresh nutrients to maintain differential corrosion cells.

The development of high gas pressure technology has enabled the generation of high quality definitive images of hydrated matter not before imaged with electron microscopy (Little, 1991). The gas itself contributes to the amplification of signals and so acts as a detector (Danilatos, 1991).

4.9.7.2 Scanning Confocal Laser Microscopy

Architectural analysis using SEM has been the main technique to date for studying biofilm morphology although shrinkage has been reported to occur (Woldringh, 1977). Chang and Rittamn (1986), reported the visualisation of sparse biofilms owing to the harsh sample preparation for SEM. Recent techniques such as ESEM have enabled structural analysis with hydrated specimens (Little, 1991) which ensured structural reproducibility. However, such techniques that analyse surface topology provide little information on the surface contours beneath a biofilm, nor do they allow for analysis of the viability of bacterial biofilms.

The application of a light microscope equipped with episcopic DIC and UV fluorescence to visualise the topography and viability of biofilms has been demonstrated by Keevil *et al.* (1992). However, this technique is limited by the lack of resolution at higher magnifications of 1500 x and again biofilms have to be removed to visualise the surface below. The results gained using SCLM demonstrated the potential for non invasive imaging of an intact fully hydrated living biofilm. SCLM enables optical thin sections to be analysed such that out of focus material does not interfere with the immediate image. Available from the information generated was the expansion of the horizontal image to a cross section or vertical sagittal (xz) section, providing a side on view of the specimen.

4.9.7.3 Surface analysis

Fluorescein (0.1%) was used as a negative stain to visualise the copper surface. With this technique deposits could be identified on the surface as could contours such as pits. The copper surface itself appears dark (Fig. 4.52, page 219). Comparisons were made between copper coupons from the vessel supplied with unfiltered water and the one supplied with filtered water. Variations were not evident in the horizontal direct view of the surface of the copper coupons supplied with filtered water (Fig. 4.38, page 187). When a sagittal section was generated the surface was uniform with no pits or mounds (Fig. 4.39, page 188). As bacteria were present in this system this may indicate that bacteria are not primarily responsible for the type of corrosion identified as pepper pot pitting. In contrast copper coupons that had been immersed into the vessel supplied with unfiltered had numerous mounds or pits that are out of focus and so appear black (Fig. 4.40, page 189).

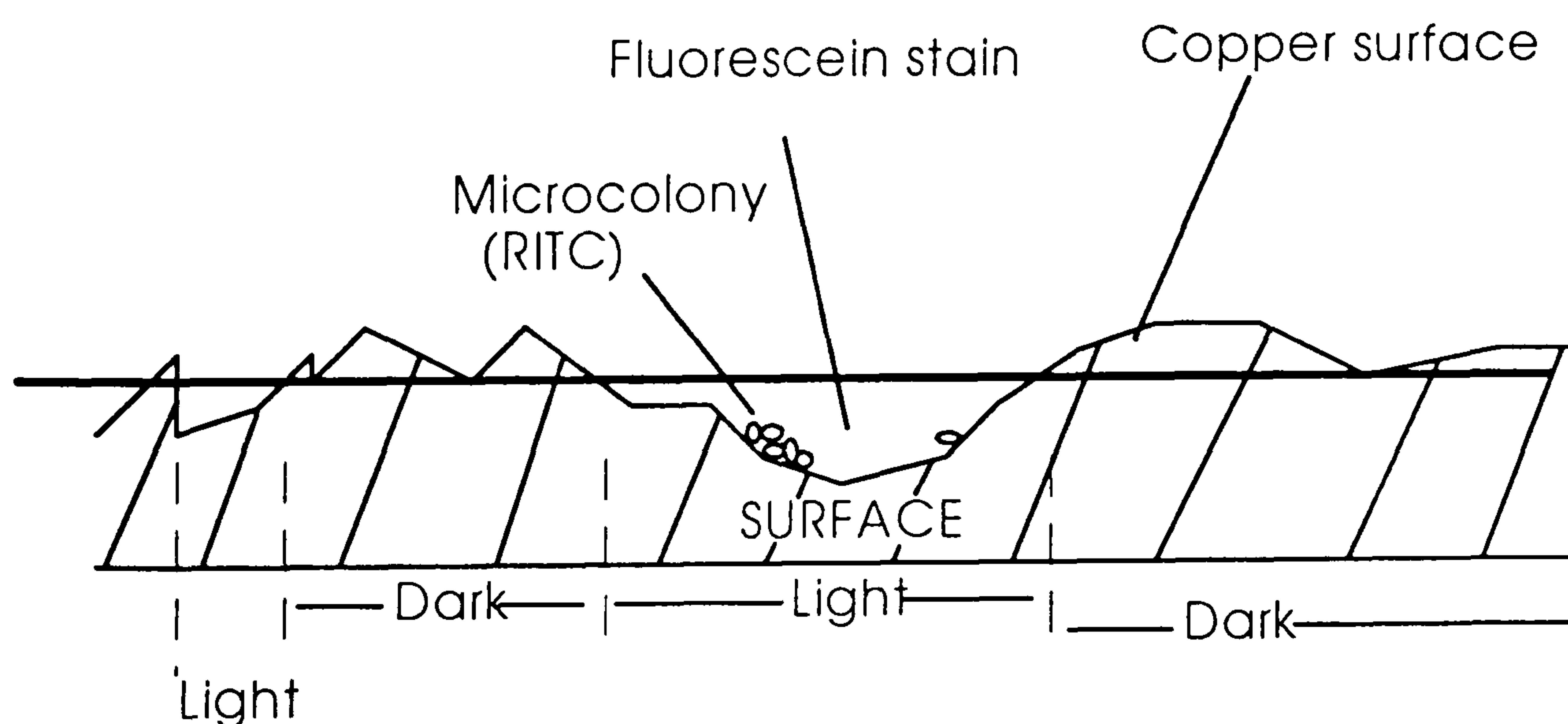


Fig 4.52. Schematic of negatively stained surface using fluorescein and RITC stained bacteria on the copper surface.

4.9.7.4 Visualisation of bacteria on the copper surfaces

On coupons from the unfiltered water, one particular pit was focused on (left hand image) with the corresponding right image demonstrating bacteria (Fig. 4.42, page 191). The association of bacteria within these areas was elucidated using RITC with images observed in both the horizontal (xy) (Fig. 4.42) and sagital xz sections (Fig. 4.43 and 4.44, page 192). By generating such images, it is possible to visualise the bacteria in and around the pit walls. Similar techniques have been utilised by Cummins *et al.* (1992) to demonstrate, using a CLSM, that the development of a *Streptococcus sanguis* biofilm was critically dependent upon the presence of *Actinomyces viscosus*. For identification of *Streptococcus sanguis* and *Actinomyces viscosus* they utilised RITC and FITC respectively.

4.9.7.5 Determination of bacterial metabolic activity

Analysis of metabolic activity of bacteria was carried out using 5-(and-6)-carboxy-2',7'-dichlorofluorescein with RITC to visualise the bacteria. The excitation and emission wavelength of 5-(and-6)-carboxy-2',7'-dichlorofluorescein decreases due to acidification as the emission intensity declines as the pH decreases. The surface topography was observed in photomultiplier 1 (left hand image) and the bacteria, positively stained with RITC, were viewed in the second photomultiplier tube (right hand image) (Fig. 4.45). When the two images were merged and colour enhanced a halo was found to surround the bacterial cells on the surface (Fig. 4.46). Therefore, darker areas immediately surrounding the bacteria could be indicative of a pH change due to acidification and hence the presence of metabolically active bacteria. Graber *et al.* (1986) demonstrated that four different fluoroprobes used to measure intracellular pH were subject to artefacts induced by self quenching and protein binding. Normally the measurement is based upon the ratio of excitation or emission at two different

wavelengths but as yet SCLM are unable to do this (Bassnett *et al.* 1990).

These images confirm the heterogeneity of biofilm associated with surfaces as has been demonstrated above with the light microscope, SEM and ESEM. Cummins *et al.* (1992) have also used a SCLM to demonstrate such a heterogeneity in the initial development of plaque biofilms. It is this heterogeneity that has been postulated to be important in the corrosion process and has also been confirmed by Bremmer *et al.* (1992), using atomic force microscopy to study hydrated biofilms on copper. Bremmer *et al.* (1992) demonstrated the difficulty in observing bacteria on copper tube surfaces that had not been polished smooth. The methods afforded by the SCLM negated such problems and therefore the results presented in this study are a truer reflection of the real environmental situation of the biofilm.

Although we cannot be certain that the pits in the copper tubes are the result of microbial colonisation and activity, these images do suggest a link between the deterioration of the surfaces and the presence of particulate material and bacteria. The involvement of particulate matter alone can be disregarded as the control institutions which maintained their hot water systems above 55°C did not suffer from pepper pot pitting. The ability of bacteria to create acidified zones on the copper surfaces was also demonstrated with this technique. The bacteria appear to aggravate and initiate pitting zones. This will provide differential corrosion cells resulting in areas under the respiring colonies becoming anoxic relative to the surrounding uncolonised areas. Thus, pitting already initiated by the presence of particulate material will be accelerated. Little *et al.* (1991) suggested that corrosion currents flow between peaks and troughs on metal surfaces and in agreement with this an explanation may be proposed that involves the association of bacteria and particulate material in the corrosion process.

In conclusion SCLM is an extremely useful, quick and simple technique for the study

of surface topology, bacterial morphology and viability without disruption, artefacts and with minimal specimen preparation. This is very important as although other techniques and instruments are excellent at the individual analysis of these properties, the SCLM enables not only the topology of the surface to be investigated but also analysis of a viable, metabolically active and hydrated biofilm to be carried out *in situ*. A significant feature of this technique is the elimination of out of focus information resulting in a more definitive examination of sample material.

4.9.8 Visualisation of copper tube surfaces; Epifluorescence, SEM and SCLM

Working with live biofilms it was extremely important for us to visualise biofilms with minimum treatment and disruption of the cells as possible. Historically biofilms are often thought to consist of a homogenous layer on a surface (Costerton *et al.* 1987) but studies by Keevil *et al.* (1989) have demonstrated that biofilms often consist of microcolonies on a surface. Ellwood *et al.* (1982) illustrated that bacterial attachment and biofilm development was preferred to growth in the planktonic phase. Ellwood and his colleagues also indicated that surface associated organisms can grow at approximately twice the rate of the same organisms in the water phase. Hicks and Rowbury, (1988) demonstrated that attachment to glass beads decreased the sensitivity of *Escherichia coli* to cupric ions while those in the water phase were killed upon exposure to divalent copper. Therefore, attachment increased survival because the surface not only enabled the bacteria to obtain nutrients but also afforded protection to bacteria from toxic ions such as copper.

Light microscopy offers a number of rapid techniques for examining bacteria e.g. Gram staining and cell counting chambers. However, it has been limited by maximum magnifications of 1000-1500 times and the difficulty in coping with opaque samples

using transmitted light although fluorescence has allowed the visualisation of microorganisms. The development of 150 times objective lenses has increased the magnification capabilities of light microscopes and episcopic differential interference has enabled microorganisms on opaque surfaces to be differentiated. Though episcopic DIC is most advantageous when the background is flat as on glass, with the bacteria observed as peaks. The visualisation of bacteria on glass surfaces using light microscopy (transmission mode) has been studied by Lawrence and Caldwell (1987) who demonstrated several novel colonisation manoeuvres such as packing, spreading, shedding and rolling, each of which was associated with a specific species from a natural stream population. However, materials used in domestic plumbing circuits and cooling towers are generally opaque with only a few of the plastic materials allowing transmissible light to pass through them, albeit with some difficulty. Even the plastics present a relatively smooth flat inner surface to view biofouling by DIC. The larger the diameter of the pipe the greater the amount of area which will be in view in the focal plane. It is in circumstances where curved pipe has to be examined for evidence of biofouling that the long focal length non contact metallurgical lenses are an advantage. For material such as copper and other metals the biofilm on the surface can be examined with DIC but the individual bacteria are difficult to discriminate on the copper surface although larger organisms such as fungi can be identified (Fig. 4.47). Due to this, fluorescent stains such as acridine orange and carboxyfluorescein have been utilised to observe the individual bacteria and microcolonies on copper (Figures 4.48 and 4.49) For counting purposes the use of microscopical techniques to obtain direct total and viable counts in a short period of time can be informative. Acridine orange (Daley and Hobbie, 1975) is a stain which has been used mainly for the enumeration of total bacterial numbers in a sample but the resulting range of colours from yellow to green of the individual bacteria does not necessarily equate to viability especially on heat treated cells at 60°C (Back and Kroll, 1991). Hobbie *et al.* (1977) suggested that the most active and dead bacteria will fluoresce red and inactive or slow growing bacteria will fluoresce green. AO detects

nucleic acids in a conjugation format with the fluorescence wavelength of AO differing according to the amount of DNA and RNA present in a cell. DNA, the double helix of nucleic acid forms a strong conjugate with AO and produces a green fluorescence whereas RNA produces a weak conjugate where the positively charged fluorochrome stacks on the negatively charged phosphoric base of the RNA and fluoresces red. Dennis and Bremer, (1974) produced evidence that in an *E. coli* cell under balanced growth conditions, 20% of the cells nucleic acid was composed of RNA and only 3% of DNA. Therefore, bacterial cells under balanced growth conditions should fluoresce Red and those slower growing cells which contain less RNA will fluoresce green. AO has been used successfully to identify intracellular from extracellular enteropathogens in Hela cells (Miliotis, 1991). On the copper pipe samples it was difficult to obtain an indication of viability as the range of colours varied. Another disadvantage of fluorescence was the quenching or fading of the fluorescence that occurs over a short time period. With the light microscope, biofilms have not been visualised as a flat, thick homogenous layer but as a patchy film for which the term 'mosaic' aptly describes the biofouling. Surface colonisation through the formation of discrete microcolonies leading to the development of the biofilm has also been reported by Korber *et al.* (1989). They observed that motility aided the spatial distribution of previously attached bacteria in comparison to non-motile species of *Ps. fluorescens*. As discussed earlier, electron microscopy has been used to obtain increased magnification of specimens in excess of the magnification and resolution of light microscopy. To combat the harsh pre-treatments of fixation and dehydration a cryogenic stage was used to freeze the hydrated biofilm. However, as observed in Fig. 4.50 of stringy biofilm and as described by Richards and Turner, (1984) even this technique produces artefacts of fibrillar networks. A demonstration of such artefacts was also displayed by Paerl, (1985), exhibiting association of bacteria and a marine cyanobacterium. One possible explanation forwarded for this artefact of a stringy biofilm could be the concentration of salts present in the water phase. As the water is removed under vacuum a concentration of the salts present in

the water phase may result in aggregation with the surface tension as a result of the molecules holding the products together as a string (Colquhoun, 1993).

Utilising the high pressurised sample chamber provided by the ESEM the bacteria can be observed to be encased in a hydrated matrix demonstrating that water channels exist within the matrix that binds the bacteria together. ESEM has a number of advantages in that there is no prior preparation of samples, no staining and where the facility is available EDAX analysis of the surface material can be carried out. However, this technique allows no analysis of bacterial viability or surface deterioration without removal of the biofilm.

4.10 CONCLUSIONS

The proposed model based upon continuous culture vessels has proven sufficient to study the colonisation of copper surfaces. Whereas, the planktonic population were able to survive temperatures of up to including 60°C the biofilms were controlled at temperatures above 55°C. Therefore, temperature has been shown to be an influencing factor in the survival and growth of the aquatic consortium. However, this biofilm controlling temperature was not able to control the planktonic population and another increase of 5°C to 65°C was required for a kill in the planktonic to be achieved. Therefore, at 60°C, survival of the planktonic population for longer periods of time than the biofilm would mean that the planktonic will reseed sections of the water system where the temperature falls below 60°C.

The continuous culture laboratory model has provided a small scale simulator of a water system in which to model the effect of physical and chemical change in the planktonic phase and/or biofilm bacteria. This was necessary as biofilm had been associated with pepper pot pitting corrosion of copper tube in the hot water system in

a hospital in central Scotland.

Initially the biofilms were unable to form above 55°C but after exposing the culture to temperatures up to 80°C bacteria were recovered from surfaces immersed at 60°C. Although biofouling was controlled at 60°C, higher temperatures than this were required to obtain a significant decrease in the planktonic population numbers.

Therefore even water systems operated at 60°C will not completely kill bacteria in the planktonic or biofilm phase but will exert control over fouling that will take place. In pipe sections where hot water flow is stagnant, such as toilet sinks and shower units that are used infrequently, dead ends or un-lagged sections of pipe will result in temperature loss or in the case of un-lagged cold pipe sections the temperature will increase (particularly in the winter when the building temperature is greater). The change in temperature will result in environmental changes that are favourable to bacterial growth that may lead to biofilms that harbour potential pathogens or lead to sites of corrosion.

A comparison of total counts against those obtained by viable plate counts indicated that not all the bacteria were recovered. This may have been due to a number of reasons including, non-viability, injury, agar incompatibility or nutrient shock. A major advantage of viable plate counts is not only the provision of an actual representation of the viable bacteria but also the population profiles under different conditions. For example the Gram negative bacteria may have dominated in the biofilm but they were succeeded in the planktonic phase by the *Pseudomonas* spp. These results have to be considered if biocide control is chosen as an option with which to eradicate biofilms where the choice of biocide used is determined by the presence of a particular species of bacteria.

Exposure of biofilms to 60°C for a short period of time, known as pasteurisation was

examined. Although this dramatically decreased the viability of biofilms that had been generated at 40°C there appeared to be a thermal resistance of bacteria that had been exposed to 60°C during maturation. However, the results indicated a possible synergistic action between copper and thermal inactivation of the bacteria in comparison to glass controls.

Citric and sulphamic acid achieved a complete eradication of biofilms. However, re-growth occurred on coupons treated with citric acid whereas those treated with sulphamic acid appeared to control re-growth for up to 50 days.

Although providing a uniform layer of carbonate on copper surfaces is known to decrease corrosion it was found that there was a greater number of bacteria recovered from surfaces immersed in culture media with the higher calcium carbonate concentration. Considering these results it would be of immense value to any establishment considering such treatments to monitor biofouling of pipe work during addition of calcium carbonate to the water system.

A number of techniques have been used to visualise biofilms in this study. Initially SEM was used with traditional dehydration and fixation with osmium tetroxide but the potential for loss and damage to sections of the biofilm led to other methods being investigated (Fig 4.5 - 4.7). A cryogenic stage was utilised to freeze and maintain the biofilm specimen intact but this resulted in a fibrous network and as suggested this may be due to a concentration effect of salts and organic matter.

To overcome potential loss and shrinkage of biofilm an ESEM was used to view the sample. Advantages of this technique are that wet samples can be placed into the specimen chamber without prior preparation or staining. This procedure enables higher pressures, not normally obtained with a standard SEM, and so biological specimens remain in their true morphological shape. Biofilm was observed to be

greater on the samples removed from the vessel that were supplied with unfiltered water, than from the vessel where it was filtered out (Fig. 4.32-4.37). The results may indicate a synergistic role of particulate matter in the generation of biofilms on copper pipes.

Although of immense value in studying the true morphology of biofilms the ESEM also has disadvantages in that it has to be combined with some other technique if bacterial viability or corrosion is to be assessed. Copper surfaces that were immersed in the vessel supplied with filtered water were examined by SCLM and did not exhibit any signs of corrosion (Fig. 4.38) indicating that bacteria alone were not responsible for pepper pot pitting corrosion. However, corrosion was evident on the surface of those copper coupons immersed in the vessel supplied with particulate matter. Not only were bacteria present on the surface, on the walls and in the bottom of pits but they were also shown to be associated with localised acidic zones.

Copper tube sections were also examined by ESEM, SCLM and by an adapted light microscope. The light microscope was modified to provide episcopic DIC and fluorescence and demonstrated the range of flora developing on the copper surface from fungi to bacteria that appeared to be just colonising the surface demonstrating the complexity of biofilms.

Therefore the association of particulate matter, presence of bacteria and temperatures that allow prolific metabolic activity do appear to contribute to the pepper-pot pitting corrosion of copper tubing.

In summary the laboratory simulation model has demonstrated an association between temperature and biofouling in a soft water system maintained below 55°C. At temperatures above this biofilms were controlled or reduced as was found in the control hospital during the site survey. The results demonstrate a possible association

between temperature, particulate matter and bacteria in the process of pepper pot pitting. However, it must be stressed that one must be very careful when extrapolating such results to environmental situations since each water system has its own peculiarities.

CHAPTER 5

COLONISATION OF PLUMBING TUBE MATERIALS WITH *LEGIONELLA PNEUMOPHILA*.

5.1 INTRODUCTION

The aetiological agent of legionnaires' disease, *L. pneumophila* has been found to be ubiquitous in water (Flierman *et al.* 1979 and 1981). Although environmental sources of community acquired disease are largely unknown, potable water has been identified by Stout *et al.* (1992) as the probable environmental reservoir for *L. pneumophila* for 8 out of 20 patients with confirmed and community acquired legionnaires' disease. Bartlett *et al.* (1983) found that 66% of hospitals and hotels had *Legionella* spp. in their water systems. Fischer-Hoch *et al.* (1981) identified plumbing systems as a source of infection in a hospital after eliminating the air-conditioning cooling-tower facility as a factor. Many other nosocomial legionellosis cases have been associated with hospital distribution systems (Marrie *et al.* 1992). As the present study has examined biofouling of copper used in domestic water circuits (section 4.3) the investigation was broadened to ascertain the survival of this particular pathogen in the continuous culture laboratory model. Consequently the inoculum was changed to one retrieved from an outbreak of Legionnaires' disease in Cavtat in the former Yugoslavia (supplied by Dr. John V. Lee, PHLS, UK). Several materials including copper and plastic plumbing materials were chosen as the substratum upon which to compare the generation of biofilms.

Bacterial cells contain DNA and RNA and can therefore be stained for direct enumeration using fluorescent nucleic acid stains such as acridine orange (Hobbie *et al.* 1977). Staining of viable bacterial cells is often variable depending upon conditions utilised and so Zimmerman *et al.* (1978) used a metabolic marker 2-(p-iodophenyl)-3-(-p-nitrophenyl)-5-phenyltetrazolium chloride (INT) to indicate viable cells. As respiring cells possess an active electron transport system (ETS) the ETS can be used to measure the ability of bacteria to reduce the colourless water soluble metabolic indicator INT-chloride to INT-formazan, a water insoluble red dye which is

accumulated within the respiring cell. This method has been used successfully as a rapid assay for the detection of viable *Legionella pneumophila* in environmental samples (Vesey *et al.* 1990).

Previously, Wright *et al.* (1991) utilised a defined medium to study susceptibility of pure culture *L. pneumophila* biofilms to biocides. The aim of this study was to generate biofilms composed of a mixed consortia including *L. pneumophila* in filter sterilised tap water that was representative of environmental conditions where the organism was found.

5.2 MATERIALS AND METHODS

Two continuous culture vessels were established (section 2.5, page 51) and inoculated with a complex mixed microbial consortia, including *L. pneumophila*, that was obtained from an outbreak of Legionnaires' disease in Cavtat in the former Yugoslavia (supplied by Dr. John V. Lee, PHLS, UK). Biofilms were developed on and quantified from copper, polybutylene, polyethylene and cPVC surfaces at 40°C and 60°C (section 2.6 and 2.7 page 58).

5.3 RESULTS

5.3.1 Simulation of a hot water circuit at 40°C and 60°C.

Initially the culture was maintained at 40°C to monitor colonisation of copper, cPVC, polybutylene and polyethylene with a mixed consortium containing *L. pneumophila* serogroup 1. The temperature was then changed to 60°C to determine the effect of temperature on colonisation and survival in the water phase of this consortium.

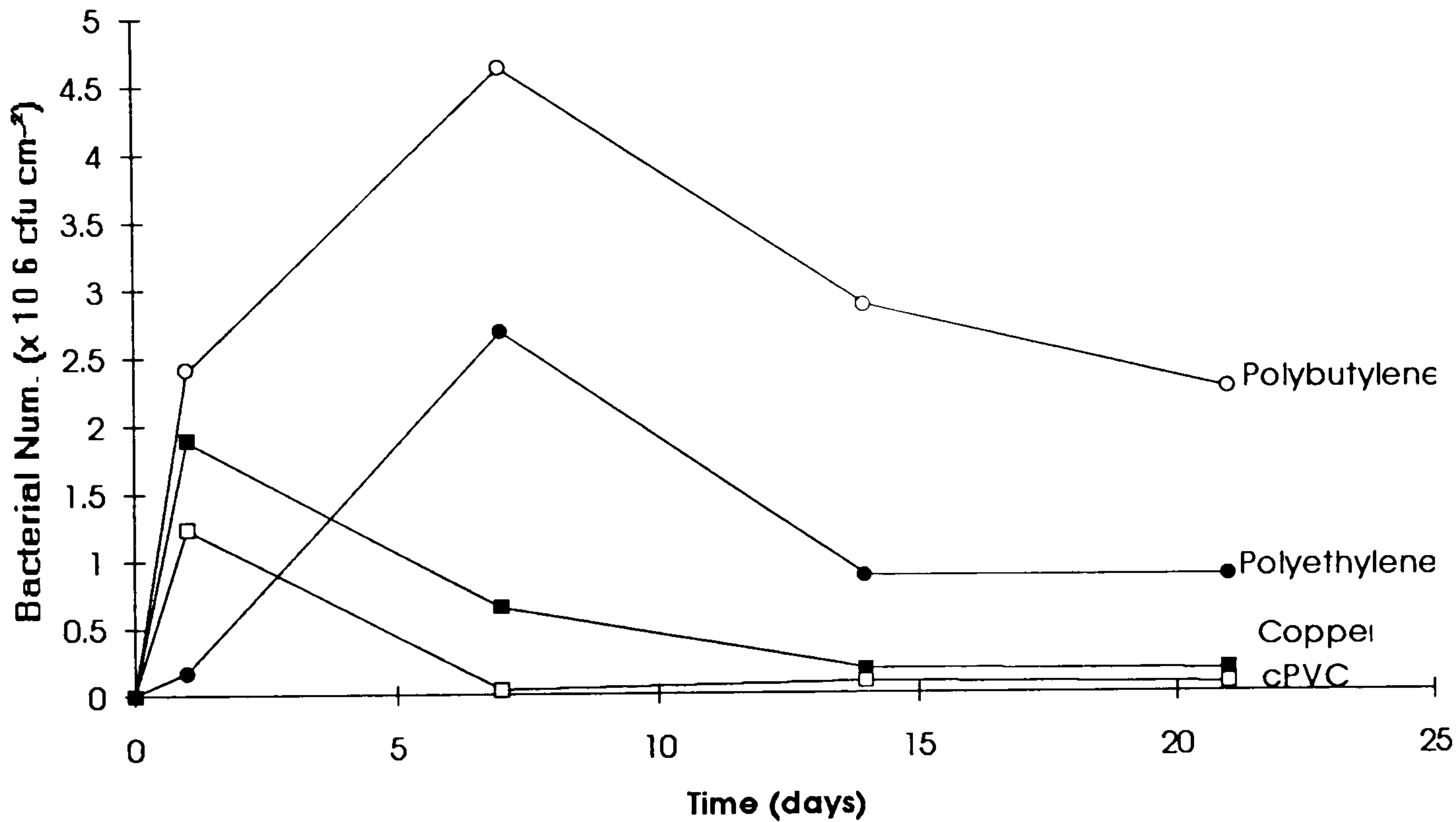


Fig. 5.1 Colonisation (total count) of copper, cPVC, polybutylene and polyethylene in Glasgow water with the Yugoslavian inoculum at 40°C.

Copper, polybutylene and cPVC were rapidly colonised in the soft water at 1.8×10^6 cfu cm⁻², 2.4×10^6 cfu cm⁻² and 1.2×10^6 cfu cm⁻² respectively at day 1 while polyethylene was only colonised at 1.7×10^5 cfu cm⁻². By day 7 the number of bacteria recovered from polybutylene and polyethylene had increased to 4.6×10^6 cfu cm⁻² and 2.7×10^6 cfu cm⁻² and then decreased to 2.9×10^6 cfu cm⁻² and 8.8×10^5

cfu cm⁻² respectively. Although bacterial numbers recovered from polybutylene decreased further to 2.2×10^6 cfu cm⁻², after 21 days polyethylene remained at 8.8×10^5 cfu cm⁻². However, from day 7 to day 21 the recovery of viable microorganisms from both copper and cPVC decreased so that there were only 1.7×10^5 and 2.0×10^5 cfu cm⁻² respectively at day 21.

5.3.1.1 Recovery of *Legionella pneumophila* from the materials in soft water at 40°C

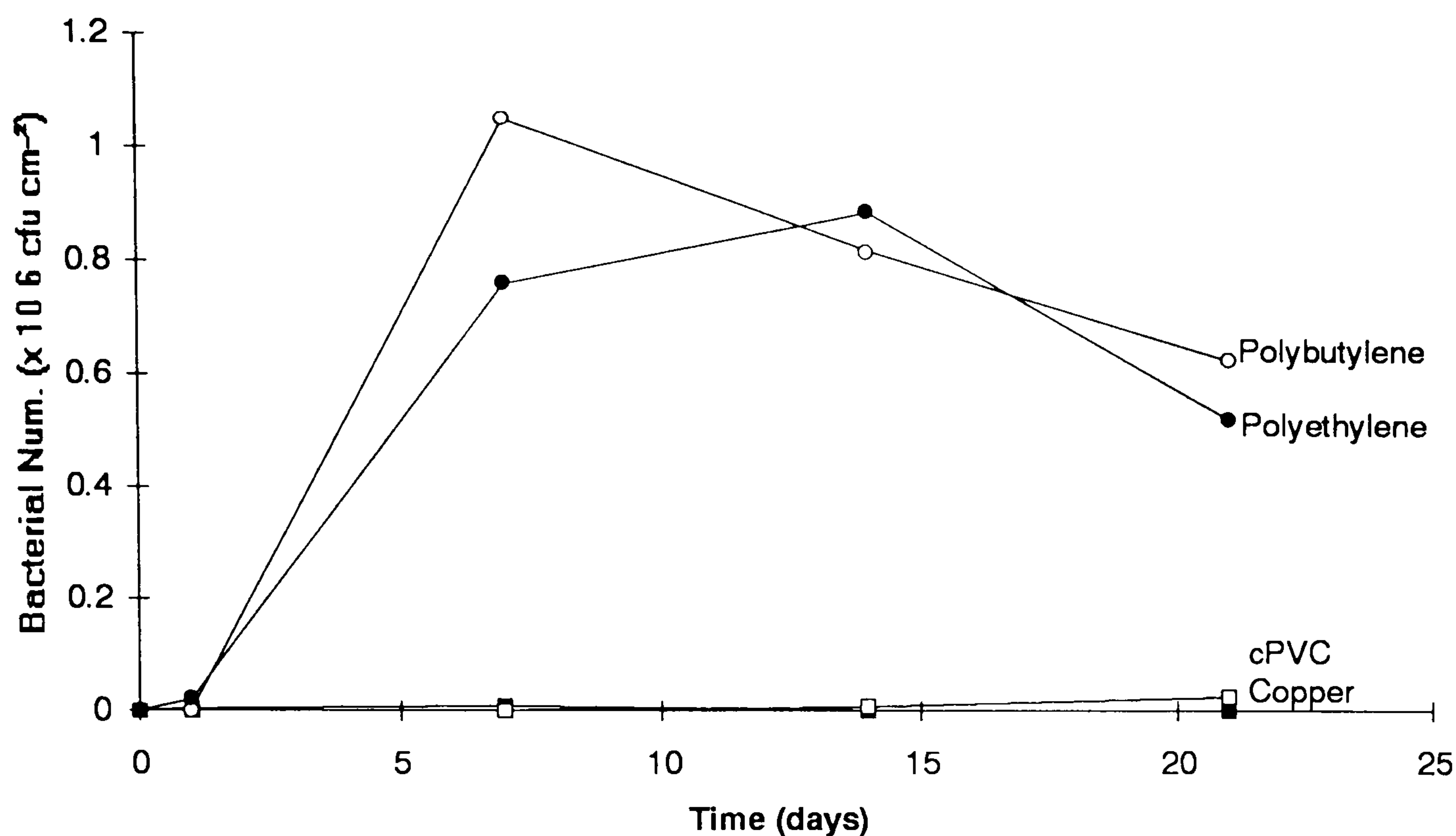


Fig. 5.2 Colonisation of copper, cPVC, polybutylene and polyethylene by *L. pneumophila* at 40°C.

At day 1 legionellae were recovered at 1.4×10^3 , 2.0×10^3 and 3.5×10^3 cfu cm⁻² from polybutylene, polyethylene and copper with no recovery of bacteria from cPVC. By day 7, 2.0×10^3 cfu cm⁻² were recovered from cPVC and 8.0×10^3 cfu cm⁻² from copper. Number of *Legionella* recovered from copper then decreased at day 14 with no recovery of *Legionella* at day 21. The number of *Legionella* recovered from cPVC increased to 7.5×10^3 cfu cm⁻² at day 14 and then to 2.4×10^4 cfu cm⁻² at day 21. However, recovery of *Legionella* from polybutylene and polyethylene dramatically increased at day 7 to 1.0×10^6 cfu cm⁻² and 7.6×10^5 cfu cm⁻² with 6.3×10^5 and 5.2×10^5 cfu cm⁻² being recovered at day 21 respectively.

5.3.1.2 Recovery of Planktonic bacteria in the soft water at 40°C.

The number of bacteria present in the water phase was also determined, while the plumbing materials were immersed in the culture.

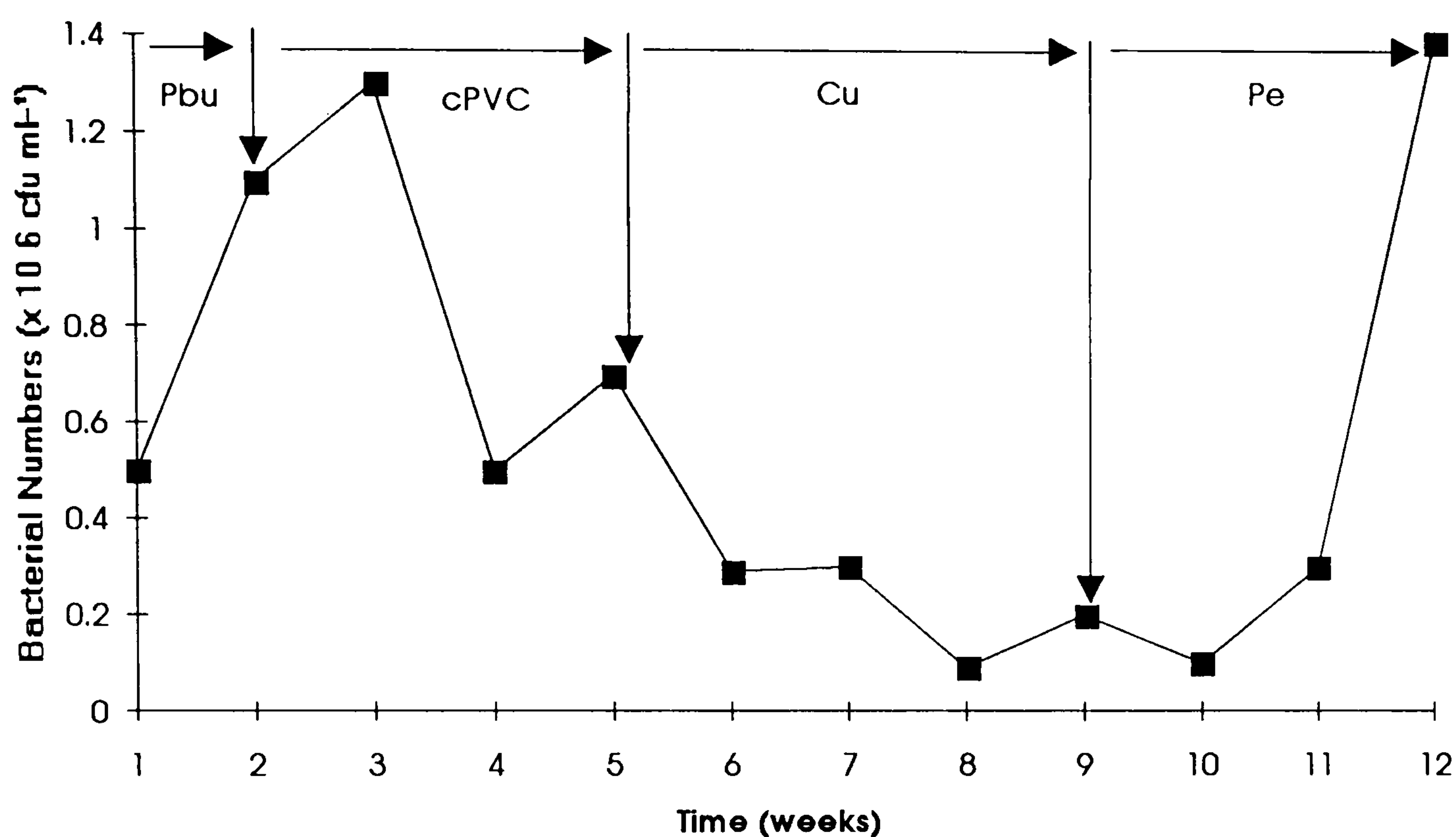


Fig. 5.3 Total bacterial numbers present in the planktonic phase of Glasgow water with Yugoslavian inoculum at 40°C.

Prior to immersion of the materials into the culture 5.1×10^5 cfu ml⁻¹ were recovered from the planktonic culture. After polybutylene coupons were immersed into the culture the number of bacteria recovered from the planktonic phase increased to 1.1×10^6 cfu ml⁻¹. Following the immersion of cPVC into the culture the number of bacteria recovered continued to increase further to 1.3×10^6 cfu ml⁻¹. The bacterial numbers decreased to 5.9×10^5 before increasing to 7.7×10^5 cfu ml⁻¹ while cPVC was immersed into the culture. Copper coupons were then suspended in the culture and planktonic bacteria recovered decreased to 2.9×10^5 cfu ml⁻¹ followed by a slight increase to 3.3×10^5 cfu ml⁻¹. Bacterial numbers in the planktonic phase then decreased further to 9.2×10^4 cfu ml⁻¹ while copper was suspended in the culture and

was at 2.4×10^5 cfu ml⁻¹ prior to removal of copper coupons. When polyethylene was immersed into the culture 1.4×10^5 cfu ml⁻¹ were initially recovered followed by 3.2×10^5 cfu ml⁻¹. Counts then increased to 1.4×10^6 cfu ml⁻¹.

5.3.1.3 Presence of *L. pneumophila* in the planktonic phase of soft water at 40°C.

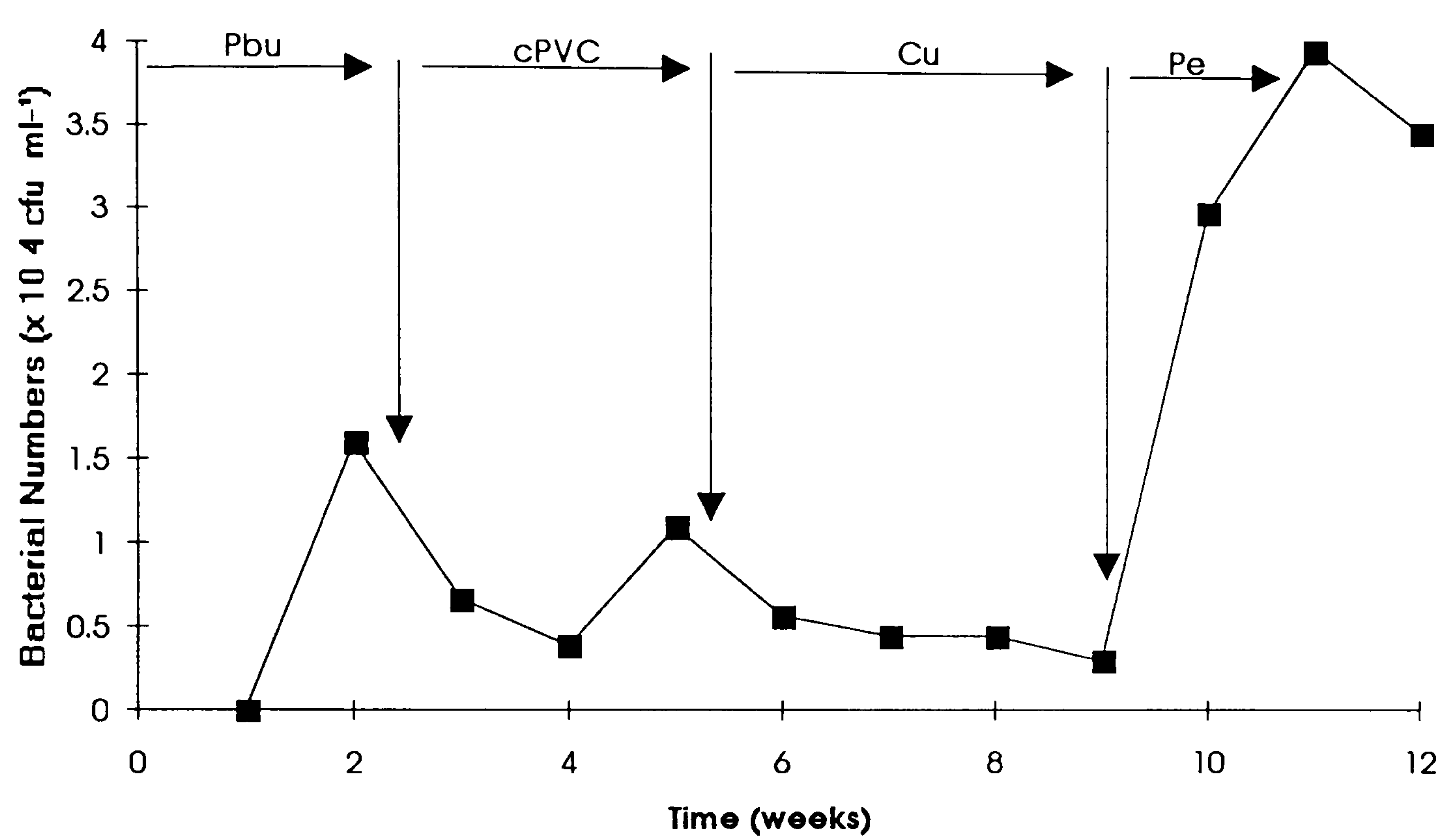


Figure 5.4 Colonisation of plumbing tube materials by *L. pneumophila* at 40°C in the planktonic phase of soft water.

Before polybutylene was immersed into the culture 20 legionellae ml⁻¹ were recovered from the planktonic phase but afterwards legionellae numbers increased to 1.6×10^4 cfu ml⁻¹. With a change of material to cPVC the numbers initially decreased to 3.9×10^3 cfu per m before increasing to 1.1×10^4 cfu ml⁻¹. When copper coupons were immersed in the culture legionellae numbers decreased consistently to 3.0×10^3 cfu ml⁻¹ and upon the introduction of polyethylene numbers increased to 3.0×10^4 cfu ml⁻¹.

5.3.1.4 Microscopical comparison of planktonic bacterial numbers and viability.

Table 5.2 Vessel 1 at 30°C.

Weeks	Total (AO)	Viable (INT)	Viable (SPC)
1	5.2×10^7	3.6×10^6	3.0×10^4
2	4.9×10^6	1.2×10^6	6.7×10^4
3	4.6×10^6	0.7×10^6	4.8×10^4
4	3.5×10^6	2.0×10^6	Confluent growth
5	6.5×10^6	2.8×10^6	2.6×10^4
6	5.3×10^6	2.6×10^6	10×10^4
7	2.8×10^6	1.0×10^6	0.8×10^4
8	4.3×10^6	0.4×10^6	3.6×10^4
9	0.4×10^6	0.02×10^6	1.0×10^4

SPC denotes standard plate count; AO denotes acridine orange; INT denotes bacterial cells enumerated by viable staining. All counts represent cfu ml⁻¹.

In vessel one, where conditions were constant, 3×10^4 cfu ml⁻¹ were recovered by viable plate counts in week 1. Whereas, using microscopy techniques, 3.6×10^6 cfu ml⁻¹ of bacteria were enumerated and identified as being metabolically active due to INT reduction. The total number of bacteria enumerated using microscopy methods (AO) was 5.2×10^7 cfu ml⁻¹ in week 1 and this does appear to be high in comparison to the other counts and may have been a dilution error. For example, week 2 is more representative of counts in this vessel, with 6.7×10^4 cfu ml⁻¹ recovered from the viable plate count and 1.2×10^6 enumerated using INT and a total of 4.9×10^6 enumerated using AO.

Table 5.3 Vessel 2 at 60°C

Time (wk.)	Total (AO)	Viable (INT)	Viable (SPC)
8	1.1×10^7	9.4×10^5	20
9	6.4×10^6	3.1×10^5	45
10	5.6×10^6	9.8×10^5	1500
11	3.0×10^6	1.3×10^5	90
12	6.8×10^6	9.1×10^5	900
13	4.7×10^6	0.8×10^5	500
14	2.4×10^6	1.0×10^6	360
15C	1.6×10^6	1.6×10^5	1100
16B	3.5×10^5	0.5×10^5	0

SPC denotes standard plate counts. All counts represent cfu ml⁻¹.

Microscopical enumeration of the culture in vessel two indicates that a total of 1.1×10^7 cfu ml⁻¹ bacterial cells were present on week 1. The total number of viable cells in the culture, identified by INT reduction, was only 9.4×10^5 cfu ml⁻¹, however, using standard plate count procedures only 20 cfu ml⁻¹ were recovered. A similar result was obtained for the other 8 weeks in which vessel 2 was in continuous mode. When the vessel was switched to batch mode there was a total of 3.5×10^5 cfu ml⁻¹ from AO and 5.4×10^4 cfu ml⁻¹ viable cells enumerated using INT. No growth was obtained using viable plate counts.

5.3.2 Simulation of hot water circuit at 60°C

Simulation of a hospital water system operated at 60°C.

5.3.2.1 Biofouling of materials at 60°C

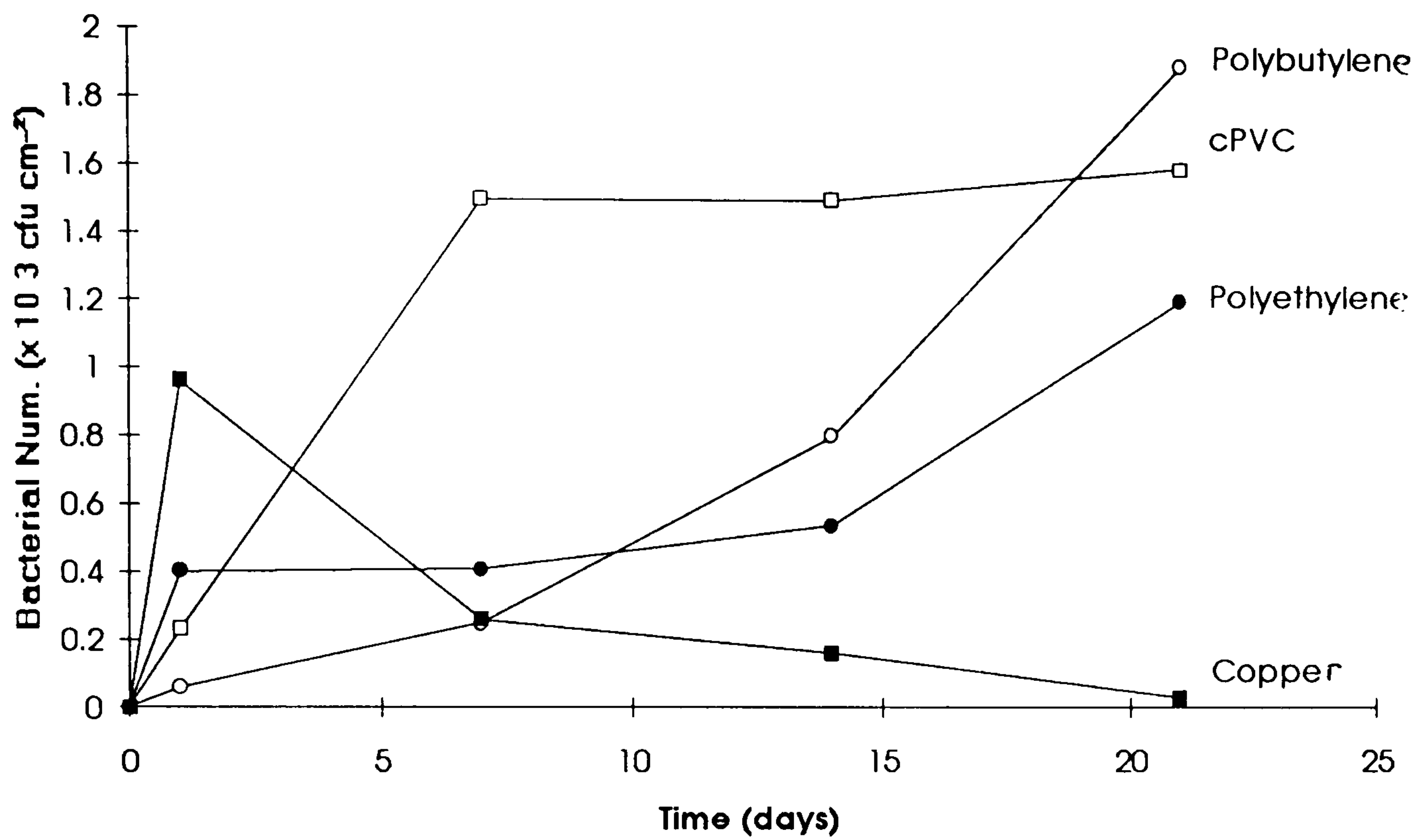


Fig.5.5 Colonisation on Copper, Polybutylene, Polyethylene and cPVC in Glasgow water at 60°C.

There were 65 cfu cm⁻² recovered from Polybutylene surfaces at 1 day, 252 cfu cm⁻² at day 7, with 800 and 1.9 x 10³ cfu cm⁻² respectively at days 14 and 21. Initially 400 cfu per cm⁻² were recovered from polyethylene at 1 day, 415 and 545 cfu per cm⁻² respectively at days 7 and 14 with 1.2 x 10³ cfu cm⁻² but at day 21 there was an increase to 1 215 cfu per cm⁻². Although only 230 cfu cm⁻² were recovered from cPVC on day 1 the numbers recovered at days 7, 14 and 21 were 1.5, 1.5 and 1.6 x 10³ cfu cm⁻² respectively. There was a total of 960 cfu per cm⁻² recovered from copper surfaces at 1 day, 260 and 160 respectively at days 7 and 14 with only 30 cfu per cm⁻² at day 21.

5.3.2.2 Recovery of *L. pneumophila* from materials at 60°C.

No legionellae were recovered from any materials while the culture was maintained at 60°C.

5.3.2.3 Recovery of Planktonic bacteria in soft water at 60°C.

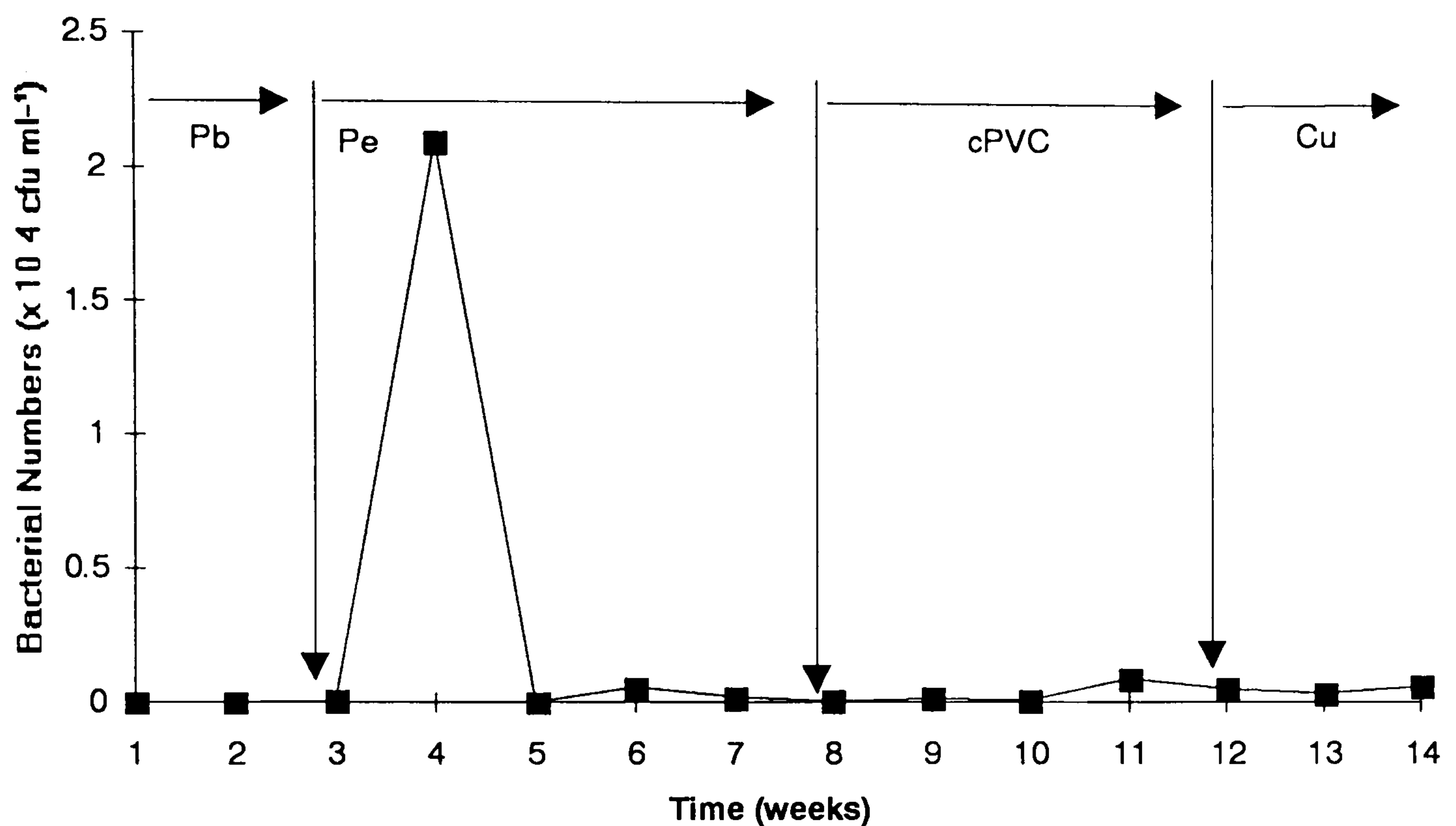


Fig. 5.6 Planktonic population at 60°C in Glasgow water with Yugoslavian inoculum.

Number of bacteria recovered at 60°C from the planktonic phase with polybutylene material immersed in the culture was less than 100 cfu ml $^{-1}$. Within 7 days of polyethylene being immersed into the culture, 2.2×10^4 cfu ml $^{-1}$ were recovered. Less than 600 cfu ml $^{-1}$ were recovered for remainder of the time that polyethylene was present. With cPVC in the culture 1.5×10^3 cfu ml $^{-1}$ or less were recovered from the planktonic phase. Copper coupons were then immersed to determine colonisation in soft water at 60°C. In this case bacterial numbers recovered from the planktonic phase was less than 900 cfu ml $^{-1}$.

5.3.2.4 Recovery of Planktonic *L. pneumophila* in soft water at 60°C.

No planktonic *L. pneumophila* were recovered from the culture maintained at 60°C.

5.3.3 Change of culture vessel from continuous to batch mode

There was an average of 587 cfu ml⁻¹ of planktonic bacteria recovered from the culture with copper immersed at 60°C. Following the extraction of copper coupons the effluent from vessel one was diverted so that it did not act as a seed for vessel two. Within 24 hours of switching to batch culture no bacteria were recovered from the vessel at 60°C.

5.4 DISCUSSION

Nosocomial legionnaires' disease has been associated with *L. pneumophila* contamination of potable hot water systems of hospitals (Wadowsky *et al.* 1982; Plouffe *et al.* 1983; Timbury *et al.* 1986 and Marrie *et al.* 1992). As legionellae have been isolated from municipal water treatment plants which adhered to accepted guidelines for water quality, treatment plants have been suspected of seeding domestic water circuits (Hsu *et al.* 1984; Colbourne *et al.* 1988a; Colbourne and Dennis, 1989). States *et al.* (1987) were able to isolate *L. pneumophila* from river water but it was not detected within any of the stages within a conventional water treatment plant. As this treatment plant maintained a chlorine residual throughout legionellae may occur there sporadically or in low numbers. However, temperatures between 20-45°C, low pressure, stagnant water, presence of metals and microorganisms are known to encourage growth and multiplication of *L. pneumophila* in water systems (Arnow *et al.* 1982; Anand *et al.* 1983; Ciesielski *et al.* 1984; States *et al.* 1984; Stout *et al.* 1985; Makin and Hart, 1991). The optimal temperature range for growth of legionellae is 35-37°C but the bacterium will grow over the range of 25-42°C (Hoge and Breiman 1991) and Makin and Hart (1991) recorded the presence of legionellae at a temperature of 50°C. In an investigation of hot water devices Alary and Jolly (1991) showed that 43% of cases reported as culture positive for legionellae also proved positive in the scrapings from peripheral outlets such as shower heads and faucet taps. Such results demonstrate the presence of biofilm as a survival niche for legionellae. Control measures such as chlorination (Cunliffe 1990) and raising of hot water temperatures (Plouffe *et al.* 1983) have contributed towards a reduction in nosocomial Legionnaires' disease.

During this study the growth of *L. pneumophila* within a mixed consortium on different plumbing materials in a simulated hot water circuit at 40°C and 60°C was

investigated. The temperature of 40°C was chosen as the minimum at which to operate a hot water system with reduced heating costs and reduced risk of patient scalding in respect to the water circuit being operated at 60°C. The higher temperature of 60°C was chosen in line with the HSS (Anon, 1990) guidelines which stipulate that hot water systems should be maintained above 50°C. A temperature lower than 55°C was not chosen as it was found (Chapter 3) that biofilms were still forming within the laboratory model operating at 55°C.

5.4.1 Simulation of a hot water circuit operating at 40°C

5.4.1.1 Biofouling of plumbing tube materials

Growth of the bacterial consortia resulted in a rapid colonisation of polybutylene, copper and cPVC within 24 hours. Bacterial numbers on polyethylene continued to increase up to 7 days exposure to the total consortia and along with polybutylene in particular continued to support greater numbers of bacteria. Due to the number of additives such as alkyl phthalates as plasticisers, butylated hydroxytoluene as antioxidant, stearates as lubricants and thioethers as heat stabilisers, some plastic pipes actively encourage the growth of microorganisms (Burman and Colbourne 1977). In comparison recovery of bacteria from cPVC and copper surfaces decreased after 24 hours.

Recovery of *L. pneumophila* from polybutylene and polyethylene at 40°C, was 100-fold greater than that recovered from cPVC, and copper surfaces. Although total numbers of bacteria recovered from the cPVC decreased after 24 hours, the number of legionellae actually increased over the time period that cPVC was exposed to the culture. Similarly there was a decrease in the total bacterial numbers and legionellae recovered from copper over the 21 day period of exposure to the consortia. No

legionellae were recovered at day 21. Recent evidence (Makin and Hart, 1990) suggests that approved listed materials (Anon, 1987) conforming to British Standard 6700, may become colonised with legionellae particularly where they are continuously subjected to temperatures which favour growth of this species. In a laboratory batch model to study colonisation of PVC, Vess *et al.* (1993) visualised biofilm as a heterogeneous mosaic of microcolonies on the PVC and was able to recover *Ps. aeruginosa* after 7 days prolonged iodopher treatment. States *et al.* (1985) cited that high concentrations of copper (10 and 100 mg l⁻¹) produced toxic effects on *L. pneumophila* and that low concentrations (0.5 and 1.0 mg l⁻¹) did not appear to be detrimental. The copper concentration of the cultures was measured by atomic absorption (results not shown) at 0.1 mg l⁻¹ and is at the low concentrations that States *et al.* (1985) suggest would not be detrimental to bacterial growth. However, in that particular study the growth of planktonic but not sessile bacteria was tested against an analytical reagent-grade copper sulphate. Hicks and Rowberry (1988) used cupric sulphate and found that concentrations of 1.5 mg l⁻¹ were capable of killing a copper resistant, plasmid-carrying *E. coli* when in the planktonic phase. But when the same cells were attached to glass beads they became resistant to concentrations of 7.5 and 15 mg l⁻¹ of divalent copper ions. In a comparison of copper and polyvinyl chloride (PVC) Bezanson *et al.* (1992) studied *in situ* colonisation using a sampling device (Robins Device) on the return end of a hot-water supply in a hospital (unfortunately the temperature of the return was not cited). They found that more legionellae were recovered more often from PVC than from copper and similar to our findings, colonisation of copper did not persist for the duration of the study. Similarly Schoenen *et al.* (1988) utilised a model hot water system and found that legionellae did not persist on copper for the duration of the experiment. In another hot water (45°C) model system, Schofield and Wright, (1984) and Schofield and Locci (1985) examined rubber and copper materials extracted after ten weeks exposure to legionellae and found that copper was the least colonised in comparison to rubber. In this present study recovery of legionellae from copper peaked at day 7 then decreased

with none recovered at day 21. These results suggest that there are factors related to the cPVC surface and more so the copper surface that inhibit or suppress colonisation by *L. pneumophila*. Colbourne *et al.* (1984) demonstrated that legionella were able to survive within a biofilm on plastic and rubber plumbing circuit fixtures and fittings despite the introduction of control measures such as chlorination and increased temperature. However, in a test protocol (Colbourne and Brown, 1979) to test microbial growth potential of water supply materials unplasticized PVC passed the test whereas plasticised PVC failed. The test was based on differences in dissolved oxygen between water samples containing materials and a control that did not.

Copper has been attributed with the ability to suppress fungi and bacteria and has consequently been used in agriculture for many years to control plant infections (Bitton and Friehofer, 1978; Cooksey and Azad, 1992). In the water industry copper has been shown to suppress not only coliforms (Domek *et al.* 1984) but also *L. pneumophila* (West *et al.* 1989; Landeen *et al.* 1989) and other microorganisms (Singh and McFeters, 1987). Copper ions are believed to interfere with enzymes involved in cellular respiration (Domek *et al.* 1984) and to bind at specific sites to DNA (Liebe and Stuechr, 1972a and b). Mechanisms of resistance to copper involve internal complexation, mainly utilised by eukaryotes (Gadd and White, 1989), while bacterial cells have been shown to efflux heavy metals such as zinc (Neis, 1992) and copper (Brown, *et al.* 1992) actively to prevent accumulation within the cell. Bacteria have also been shown to be able to complex toxic divalent cations by the production of surface active polymers (Corpe, 1975). Organisms may rely on several survival strategies, for example metallothionein synthesis is a mechanism of copper resistance in yeast but copper binding around the cell wall and transport across the cell membrane (as discussed above) may also be components of the total cellular response (Gadd and White, 1989).

5.4.1.2 Growth in the planktonic phase at 40°C

Total bacterial numbers in the planktonic phase doubled to $> 1 \times 10^6$ cfu ml⁻¹ when polybutylene surfaces were immersed in the culture. With cPVC 5.9×10^5 cfu ml⁻¹ were recovered but after the immersion of copper coupons the planktonic flora decreased to 9.2×10^4 cfu ml⁻¹. Bacterial numbers recovered from the planktonic phase was 1.4×10^5 cfu ml⁻¹ after the immersion of polyethylene and then exhibited a 2-fold followed by a 10-fold increase in recovery. Therefore with copper present in the culture the number of recovered bacteria decreased. Recovery of legionellae from the planktonic phase followed a very similar pattern to that of total numbers indicating that there may be a link in the number of legionellae to the total population. The decrease in total planktonic bacteria with copper present in the planktonic phase was 8.3-fold while legionellae exhibited a 3.6 fold decrease. With polybutylene the number of legionellae exhibited an 800-fold increase in numbers, a 10-fold increase in the presence polyethylene, and only a 2.8-fold increase in the number of legionellae with cPVC present in the culture.

These results indicate that plastic materials polybutylene, polyethylene and to a lesser extent cPVC encourage growth of not only total numbers but also legionellae within the planktonic phase. In a study of the microbial growth potential of materials in potable water, Colbourne (1985) stated that materials donate organic compounds either through leaching or by exposure at the surface. From Fig. 5.3 it appears that cPVC may indeed leach nutrients into the water phase rather than at the material surface. The growth at surfaces of certain plastics materials can be self perpetuating, e.g. polybutylene and polyethylene (Fig. 5.1 and 5.3). This would not only support biofilm growth but also result in increased microbial activity in the water phase (Colbourne, 1985). In a study of the assimilable organic carbon to determine the potential of a water to support growth van der Kooij *et al.* (1982) demonstrated that a much higher AOC was produced in the presence of plasticised PVC. Bezanson *et al.*

(1992) also cultured the water flowing through an *in situ* copper sampling device and recorded numbers ranging from the equivalent of 200-1000 cfu ml⁻¹. In comparison numbers recovered from the planktonic phase of the present study using filter sterilised tap water ranged from 20-40000 cfu of legionellae ml⁻¹. This represents a 40-fold increase on the maximum obtained by Bezanson *et al.* (1992). In contrast, Wright *et al.* (1991) who utilised a defined nutrient media with added nutrient sources, obtained 1.2×10^7 cfu of legionellae ml⁻¹. This is a 300-fold increase on the maximum recovered from the planktonic phase of the continuous culture model using tap water as the nutrient source and results in artificially high legionellae numbers not usually encountered in potable water systems.

5.4.2. Simulation of a hot water system operated at 60°C

As well as operating the continuous culture model to simulate a hot water system maintained at 40°C the model was also operated at 60°C according to guidelines (Anon, 1990) for the control of legionellae. Total fouling was dramatically reduced in comparison to that at 40°C e.g. maximum colonisation of polybutylene was 4.6×10^6 cfu cm⁻² at 40°C while at 60°C 1900 cfu cm⁻² were recovered. There was also a different profile of fouling for the three plastic plumbing materials. Bacterial numbers recovered from polybutylene and polyethylene increased the longer the coupons were immersed into the culture and was still increasing at day 21 when 1900 and 1215 cfu cm⁻² were recovered respectively. By day 7 the number of bacteria recovered from cPVC had reached 1600 cfu cm⁻² and was maintained at this number up to and including day 21. Although 1000 cfu cm⁻² were recovered from copper surface within 24 hours the numbers of bacteria recovered continued to decrease such that only 30 cfu cm⁻² were recovered at day 21. Thus at 60°C copper plumbing tube material continued to suppress biofilm growth and may act in synergy with thermal pasteurisation as indicated in chapter 3 section 4.5 to control fouling. The maximum

number of bacteria recovered from the plastics was only $> 1 \times 10^3$ cfu cm² with polybutylene, polyethylene and cPVC exhibiting increased colonisation in comparison to copper.

5.4.2.1 Growth in planktonic phase at 60°C

In contrast to $>1 \times 10^6$ cfu ml⁻¹ being present when the culture was maintained at 40°C, less than 100 cfu ml⁻¹ were recovered initially at 60°C. Although 2.2×10^4 cfu ml⁻¹ were recovered while polyethylene was present in the culture this was not representative of the bacterial numbers recovered at this temperature and may have been due to an aggregate of cells from vessel one or a heating element failure. With the temperature maintained at 60°C the maximum number of bacteria recovered from the planktonic phase was 150 cfu ml⁻¹ and at all sample points fewer bacteria were recovered.

Other studies have demonstrated legionellae control using temperatures $>55^\circ\text{C}$. Plouffe *et al.* (1983) found that in a survey of six buildings only two were consistently negative for *L. pneumophila*. The hot water storage temperature was maintained at 43-45°C in both these buildings, whereas the other four buildings which were negative for *L. pneumophila* maintained their hot water between 58-60°C. Such results clarify the importance of thermal inactivation of bacteria particularly for *L. pneumophila*. Dennis *et al.* (1984b) reported that in batch culture at 50°C and 54°C *L. pneumophila* survived longer than a coliform, *Pseudomonas* spp. and a *Micrococcus* spp.. Similar results were obtained by Stout *et al.* (1986) at 60°C between *L. pneumophila* and a *Pseudomonas* spp. These results indicate that relatively high temperatures are likely to favour the growth of legionellae at the expense of other environmental organisms. In a risk assessment of domestic hot water systems Alary and Joly (1991) reported that legionellae were recovered from 39 % of hot water systems where hot water was supplied by an electric heater, with no recovery of legionellae where hot water was

supplied by oil or gas heater. In the latter the heat source is normally located at the bottom of the heater and so even when sludge does accumulate it is maintained at too high a temperature for legionellae survival. Electric calorifiers (heating element located on the side of the electric heater) operated at recommended temperatures of $>55^{\circ}\text{C}$ have been implicated as reservoirs for multiplication and growth of *L. pneumophila* (Bhopal and Barr, 1991). This is due to an accumulation of sludge and the presence of cold water spots below the heating element protecting microorganisms and allowing growth to occur at $30\text{-}40^{\circ}\text{C}$. Stout *et al.* (1986) suggested that thermostat set points be set at 65°C to 70°C to achieve a temperature of 60°C at the bottom of calorifier tanks and at outlets. In a study of mechanisms of control of *L. pneumophila* Muraca *et al.* (1987) used a model plumbing system (closed circuit but flowing) to demonstrate that 20 minutes at 60°C was required to achieve a 1 log drop in numbers of surviving *L. pneumophila*. Using heat inactivation a 5 log kill was achieved quicker than with chemical mechanisms of inactivation such as chlorine and ozone.

Heimberger *et al.* (1991) reported that thermal pasteurisation of a hot water system should not be relied upon to completely control legionellae. In a hospital where the hot water system was maintained at 50°C , the complete water system was exposed to hot water flushing at 75°C for 15 minutes at all outlets and chlorination of the hot water tanks. However, after 1 month *L. pneumophila* was cultured from several outlets and only continual chlorination of 1.5-2.0 ppm and regular heat flushing of water tanks prevented legionellae from multiplying in the water circuit. Howells *et al.* (1986) also reported an outbreak of nosocomial legionnaires' disease in a hospital eight weeks after the hot water temperature of $55\text{-}60^{\circ}\text{C}$ was decreased by 10°C . Such results indicate the tenacity of this bacterium to survive, perhaps in a biofilm in the water circuit (Colbourne *et al.* 1984) and to proliferate when conditions become favourable. The survival and recalcitrance of *L. pneumophila* may be due to its growth within a biofilm as suggested above or it being associated with other bacterial

species, protozoa such as *Tetrahymena* sp. (Barbaree *et al.* 1986) and amoebae (Rowbotham, 1980) such as *Acanthamoeba polyphaga* (Barker *et al.* 1992). There is no doubt of the co-cultivation and multiplication of *L. pneumophila* within free-living amoeba (Anand *et al.* 1983) which are ubiquitous in water and have actually been isolated from the laboratory model (Rowbotham, 1991). However, States *et al.* (1993) have recorded that multiplication of *L. pneumophila* within *Hartmonella vermiformis* does not significantly increase heat resistance of *L. pneumophila* to short term exposure to heat.

Over the last two decades there has been a change in guidance for control of temperatures in hospital hot water systems which has resulted in a reduction of the temperature from 60°C to between 40°C and 45°C. Although this change was initially introduced to conserve energy it was later (in the USA) made a mandatory regulation that temperatures should not exceed 43°C to minimise scalding of patients in hospital wards (Joint Commission of Accreditation on Hospitals, 1981) - a similar guideline was followed in the United Kingdom. In 1983 the Joint Commission of Accreditation allowed each hospital to determine its maximal hot water temperature. Therefore although the use of high temperatures (60°C) have been shown to eradicate legionellae from the laboratory model, *in situ* hospital staff have to be aware of the danger of scalding as temperatures of 60°C can cause partial thickness burns with a contact time of 5s (Makin and Hart, 1991).

From microscopical examination of planktonic bacterial counts in vessel two at 40°C the viable plate counts are 100-fold lower than the viable INT count of which the total number of bacteria present on the filter as determined by AO counting is >2-fold. Liebert *et al.* (1983) found that culturable counts were only approximately 10-fold lower than direct microscopic counts using AO. In the second vessel at 60°C the total (AO) bacterial numbers were >5-fold more than the viable numbers (INT) however, number of bacteria detected by viable plate counts was less than 1500 cfu ml⁻¹.

When the materials trial was completed the effluent flow from vessel one was diverted and ran to waste, such that vessel two only received sterile media and was not seeded with spent cells and media. This was carried out to ascertain the survival of planktonic bacteria at 60°C. Within 24 hours no viable bacteria were recoverable from the planktonic phase by agar plate culture, yet 5.4×10^4 cfu ml⁻¹ still had an active electron transport system that was capable of reducing the metabolic indicator INT. When the culture temperature was maintained at 60°C and was not seeded with effluent from the vessel at 30°C no bacteria were recovered from the planktonic phase within 24 hours. When the culture was reseeded with the effluent from the vessel at 30°C the population was recoverable by viable plate count indicating that, when constantly challenged with a bacterial consortium, 60°C may control but is unable to kill the bacteria. This has importance in hot water systems where the temperature in parts of the system may fall below 60°C and so provide a favourable environment for multiplication and growth of bacteria which will reseed the rest of the system. Extracted formazan has been measured spectrophotometrically to quantify electron transport activity (Blenkinsopp and Lock 1990) but this was not a suitable method to use in these experiments as the volume of biomass was not large enough (results not shown). Fry and Zia (1982) suggested a relationship between cell size and viability with the possibility that small cells could be dormant (Stevenson, 1978) and therefore unculturable (Hoppe, 1976). Torrela and Morita (1981) discussed that heterotrophic bacteria may have evolved mechanisms for survival in being resistant to starvation by becoming very small. Small cells would have a minimum nutritional requirement and turnover of cell components but may still be unculturable. Such mechanisms may account for the number of bacteria which appear to be viable according to the results of the microscopy (INT) but may have been injured to such an extent that they did not survive the trauma of culturing on agar. Arguably this may also have been the case for the legionellae but immunofluorescence labelling and metabolic indicators were not utilised in this particular study to see if legionellae were still present and or viable in the aqueous phase of the culture. Those injured and hence unculturable bacteria

may have recovered under more favourable conditions to form another bacterial population of aquatic and biofilm bacteria including legionellae.

5.5 CONCLUSIONS

The growth of legionellae in the environment is of major concern to those responsible for the public health. This applies particularly in hospitals where many patients are immunocompromised and are therefore more susceptible to infections. However, the publicity which is attracted by Legionnaires disease means that it attains a high profile in the eyes of the public and those responsible for their health to the extent that other issues which may be as serious may be put aside. Therefore, much emphasis and effort is placed upon not only detecting this waterborne pathogen but also in its control.

From this study *L. pneumophila* can grow in water as part of a mixed consortium and will colonise a range of materials including copper and cPVC, both of which appear to suppress legionellae growth in comparison to other plastic materials at 40°C. A material that suppresses the growth of legionellae, however, is not sufficient to control its growth or the consortium at 40°C without some other form of physical or chemical control.

Increasing the temperature of the model system to 60°C clarified that this will control the presence of legionellae and the consortium on the surfaces of materials particularly in the presence of copper. The planktonic bacteria were however, able to survive at 60°C as indicated by INT and would potentially colonise a surface downstream where conditions were more favourable. This may also apply to injured and unculturable legionellae. In many studies of hot water systems the use of temperatures of 60°C and above in the calorifier and at all outlets have been shown to eradicate legionellae to prevent nosocomial legionnaires' disease. However, when the temperature has been returned to less than 50-55°C the recovery of bacteria may occur resulting in potential cases of legionnaires' disease. Temperature control has only been temporary

and deemed to fail as there is no residual effect on the bacterial population after the temperature has been reduced to less than 55°C.

Alternative mechanisms such as chemical treatment have to be cost effective and since a residual exists in the water system they can be operated at less than 50°C which also helps to prevent cases of patient scalding. However, the environmental issue of disposal is also of importance and has to be considered.

Where temperature is used as the method to control legionellae within hot water systems maintaining the temperature at or above 60°C would only control legionellae if the temperature is rigorously maintained at all faucet and shower outlets.

Maintenance and control of any mechanisms employed to control legionellae must be strictly adherent to the guidelines recommended otherwise this tenacious persistent human pathogen will continue to re-emerge.

CHAPTER 6.0

CONCLUDING DISCUSSION

6.1 BACKGROUND

The process of corrosion involves many biological and environmental factors. Pepper pot pitting corrosion observed in this study did not entirely lie within the boundaries of classical types of corrosion, so it has been difficult to explain. In this concluding discussion a proposal involving many different aspects of bacterial growth and corrosion have been drawn together to provide a theoretical mechanism of pepper pot pitting. Attachment of bacteria to surfaces has been shown to enhance not only their survival but also their growth (Costerton Geesey, 1979). Hicks and Rowbury (1988) demonstrated that attachment to glass beads decreased the sensitivity of *Escherichia coli* to cupric ions while those in the water phase were killed thus attachment to a surface increased survival. Therefore in that study formation of a biofilm was an advantage for survival and perhaps similarly for water borne bacteria and pathogens in water systems. Problems in water systems due to biofouling such as microbially induced corrosion of substrata have been well recorded and documented (Iverson, 1987). Pitting corrosion of copper by classical mechanisms has been classified into three typical corrosion mechanisms

Type 1 Corrosion

Type 1 pitting occurs in cold hard water supplied from bore holes and the pitting propensity depends on pH, dissolved oxygen, chloride, sulphate, sodium, nitrate concentrations, low organic carbon and high dissolved inorganic ions. The pits contain soft crystalline cuprous oxide and cuprous chloride under a film of cuprous oxide crystals covered by basic copper carbonate (Fig. 6.1). Campbell (1950) reported that pitting corrosion of copper from cold water circuits was due to the presence of a glossy black film found beneath the corrosion carbonate scale. Under X-ray analysis the film was found to be composed of 2% copper and predominantly an amorphous

substance of very low X-ray absorption such as carbon. The carbon residue may have arisen from a) graphite in the extrusion lubricant, b) breakdown of lubricant during drawing and c) cracking of residual bore lubricant during the bright annealing operation (Cornwell *et al.* 1973) but was basically a result of fabrication.

Two mechanisms were proposed for the effect of carbon scale on copper. The first was that the carbon scale would act as an efficient cathode acting in conjunction with smaller anodes where the carbon film was broken. Alternatively the carbon film may act as a concentration cell creating differentials of oxygen with the production of a large number of small pits. The failures reported by Campbell (1950) were unusual as they occurred in pipes with a service of less than 2 years. The above author also determined that the extent of corrosion increased as the quantity of carbon on the copper surface increased and suggested that the source was residual lubricant from fabrication of the tubes. There appeared to be a questionable relationship in both cold and hot water circuits as the deposits in hot pipes usually contain silt as bulky residue which were unidentifiable from carbon scale (Campbell, 1950).

Cornwell *et al.* (1973) examined carbon scale on copper pipes and found that the pitting was induced by a hard water. Chemical analysis of the water supplying those particular water circuits in Scotland with failed copper tubes suggested a water type similar to that used by Cornwell *et al.* (1973) as a control for a non-pitting water i.e. soft water with relatively low total dissolved solids at 180°C. Interestingly the non-pitting water used by Cornwell also had a high organic matter content as measured by the method of Campbell (1954).

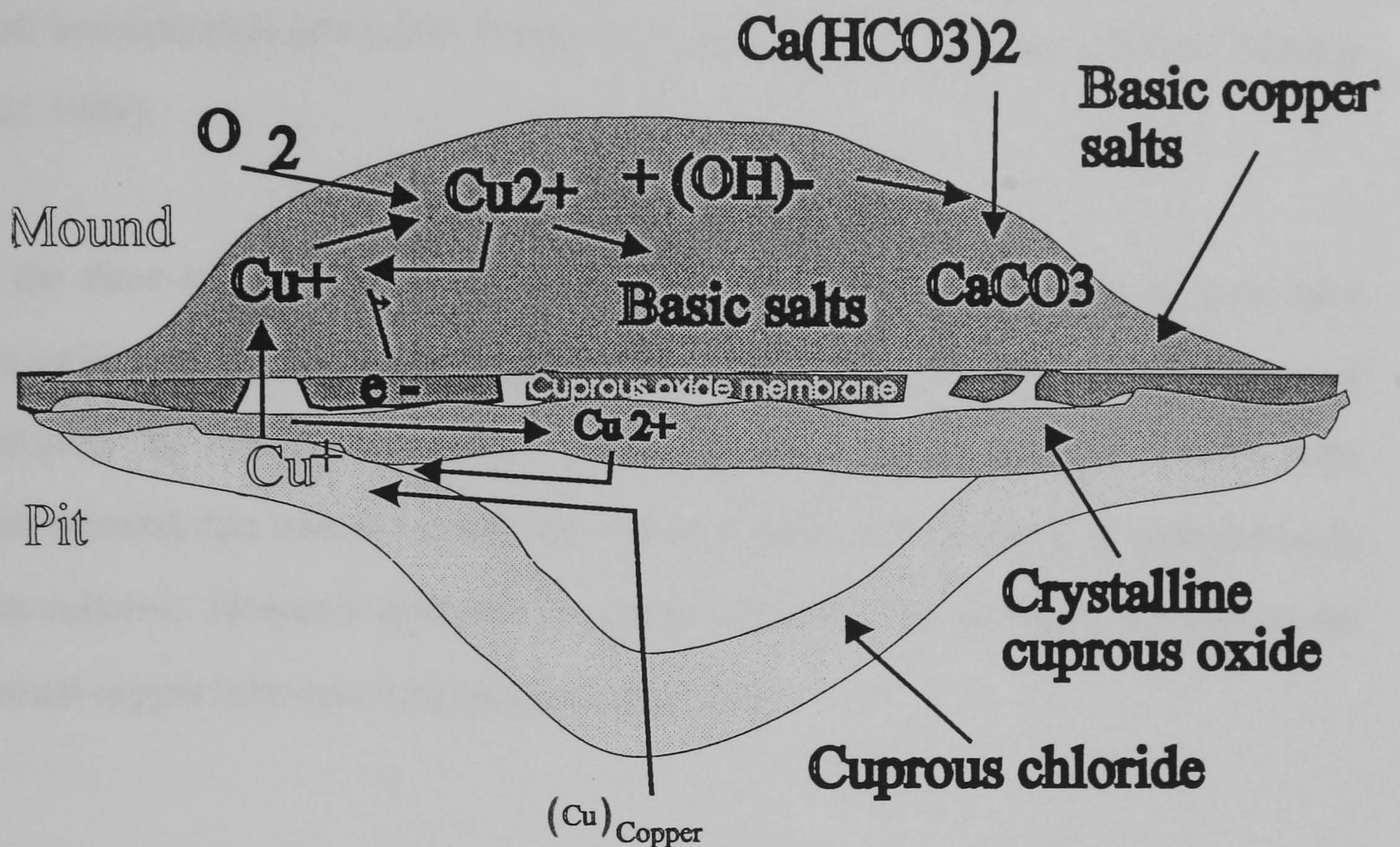


Figure 6.1 Diagrammatic representation of the arrangement of corrosion products and of the reaction involved in pitting corrosion of copper. (Lucey 1967)

Type 2 Corrosion

Type 2 pitting (Holm *et al.* 1962; Mattson and Fredrikson, 1966). Pits contain crystalline cuprous oxide and are covered by tubercles composed of copper oxides and basic copper sulphate. An adherent cupric oxide/cuprous oxide layer exists over the unpitted surfaces. Characteristically this type of pitting only occurs in the hottest section of hot water circuits where the temperatures are typically in excess of 60°C.

Type 3 Corrosion

Type 3 pitting is infrequent and occurs in pipes carrying cold water with a high pH, low hardness, low mineral and low organic content where the pits are characterised by small hemispherical pits under a common covering of basic copper sulphate (Shalaby *et al.* 1989).

Of the three types of copper pitting that have so far been discussed all have been categorised with recognised mechanisms. The promotion and acceleration of corrosion by bacteria in copper alloys was reported by Rogers (1948) who demonstrated that mixed cultures resulted in a more severe attack in comparison to pure cultures. However there did not appear to be any link between bacteria and the unusual copper tube corrosion until more recently.

6.2 INTERACTION OF BIOFILMS IN PEPPER POT PITTING CORROSION

The type of pitting which occurred in copper pipes from the water systems in Scotland resembled Type 1 corrosion in that pits were hemispherical and contained soft crystalline cuprous oxide with varying amounts of cuprous chloride under a cuprous oxide membrane. The pitting also resembled Type 2 corrosion as oxide on the surface between pits was largely cupric with tubercles above the pots being basic copper sulphate often with a cupric oxide on part of the deposit itself. Therefore it is a combination of both Type 1 and Type 2 corrosion.

In the soft water plumbing circuit I have hypothesised how the biofilm develops and becomes involved in the corrosion process described as pepper pot pitting. Environmental aquatic systems contain organic matter as dissolved organic material (DOM), in the range of 25-72% with a mean of 42% (Reuter and Perdue, 1977). Humic acids account for 50% of this group and are thought to be refractory, relatively

unknown hydrophilic acids (30%) and the simple compounds such as carbohydrates, carboxylic acids, amino-acids, hydrocarbons. Hydrophilic acids are thought to be composed of volatile fatty acids and hydroacids as well as complex polyelectrolyte acids that probably contain many hydroxyl and carboxyl groups. Such hydrophilic acids would certainly support microbial growth. Therefore it would appear that 50% of dissolved organic carbon, termed the assimilable organic carbon may be able to support microbial growth. AOC, of the soft upland Scottish water was four times higher than that found in harder lowland river waters.

Dissolved organic matter, present in the soft water may form a pellicle on the copper surface providing a buffering region for the initial contact of bacteria onto the copper surface (Fig. 6.1 (1)). Bacteria will be transported from the water phase by convection but once in the 40 μm viscous sublayer active transport will drive them to the surface where they will come into contact with this initial pellicle. At the surface, bacteria will be limited to utilising DOC in the pellicle as a nutrient source. In the viscous sublayer convective transport of nutrients will be zero and the movement of substrates will be under the control of molecular diffusion. Bacteria will also be protected from the Cu^{2+} ions which have complexed with organic matter (Fig. 6.1 (2)). For bacterial cells that come into contact with Cu^{2+} ions they may use a mixture of defence mechanisms from chelation which may be internal (Gadd and White, 1989) or external such as exo-polysaccharides (Marszalek, 1979) and/or exclusion (Rouch, 1985). Formation of microcolonies (Fig. 6.1 (3)) will lead to differential concentration cells caused by varying concentrations of oxygen, acids (metabolic acids) and exopolysaccharides (Fig. 6.1 (4). Bacteria present in tubercles on surfaces of copper pipes removed from hot water circuits of buildings suffering from pepper pot pitting and from the laboratory model suggest that bacteria had found a niche in which to survive.

6.2.1 Bacterial Metabolism

Site surveys indicated that copious biofilms contained greater numbers of organisms that correlated with increased likelihood of corrosion. As bacteria were recovered from the copper tubes they were obviously not killed by copper ions suggesting copper resistance mechanisms. Although this does not suggest the cause it does indicate a relationship. This increased copious layer linked with greater corrosion rates is not unsimilar to the study of abiotic corrosion by Campbell (1950) who demonstrated that the extent of corrosion increased as the quantity of carbon on the copper surface increased. Using the laboratory model it was possible to simulate environmental conditions under which this particular type of corrosion was occurring and to form biofilms at temperatures up to and including 55°C. With the use of the SCLM (section 4.7.2) these bacteria have been shown to be present in pits as well as on the copper surfaces and are responsible for production of localised acidified zones. Bacterial metabolism results in the secretion of organic acids during fermentation of organic substrates. The rate and type of corrosion would depend on the species of organisms and the available substrate e.g. hydrogen sulphide from *Desulfovibrio desulfuricans* and sulphuric acid from *Thiobacillus thiooxidans*. Such metabolic products may result in a physical shift in corrosion as they are trapped at the bacterial metal interface. Organic acids of the TCA cycle such as citrate and fumarate were able to form metallic acids under aerobic conditions when incubated with copper, tin and zinc (Burns *et al.* 1967). Amino-acids such as dicarboxylic acids may also be aggressive to copper (Gordon *et al.* 1983). However copper surfaces present in filtered soft water in the laboratory model (section 4.7.1) did not exhibit corrosion or pitting which only occurred when both bacteria and particulate matter was present (section 4.4). When examining corrosion of carbon steel, Pope (1992) demonstrated that when microbial community members (*Enterobacter* spp., *Clostridium* spp. and *Desulfovibrio* spp.) were grown together the production of acetic acid was 10 times greater than when they were grown in pure culture. This indicates the efficacy of co-

cultivation of bacteria as has been carried out in the present laboratory model. Pope (1992) also reported that organic acid production and pitting were only significant under low nutrient conditions, again supporting the role for a laboratory model where the sole carbon source is tap water.

Results with the DIC, fluorescence, SEM and ESEM presented images of the biofilm as a heterogeneous patchy film like a 'mosaic' over the surfaces. This is in contrast to the thick $300\mu\text{m}$ film formed in the model by Wimpenny (1988) developed for physiology studies. Surface colonisation through the formation of discrete microcolonies in biofilm development has also been reported by Korber *et al.* (1989). With the SCLM I was able to demonstrate that individual bacteria can be associated with the formation of localised acidified zones on the copper surface.

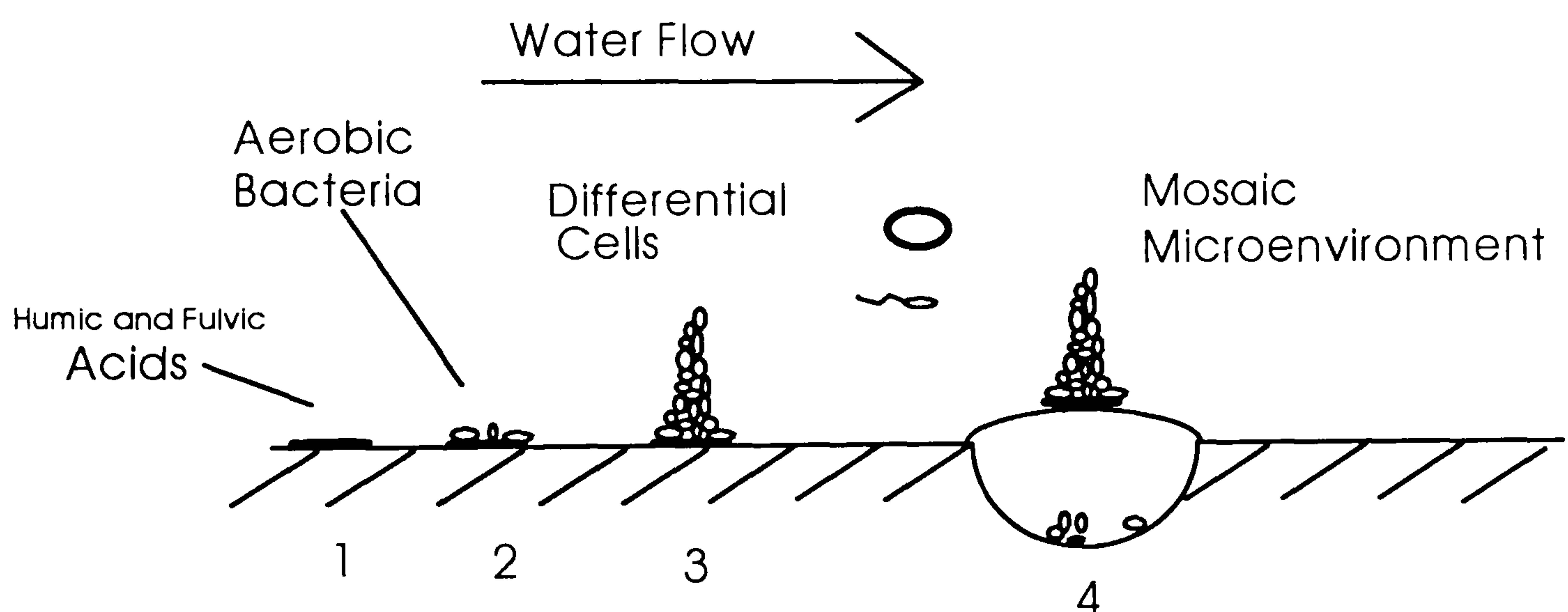


Figure 6.1 Schematic of microcolony formation resulting in sites of pitting corrosion.

(1) Formation of pellicle on the metal surface resulting in passivation. (2) Deposition of bacteria to form differential concentration cells (oxygen, EPS, acids). (3) Growth of microcolonies to form local anodic and cathodic sites through the production of metabolic products as well as chelation of copper and metallic divalent compounds by the exopolysaccharides of the bacteria. (4) Interaction of microcolonies and biofilm

with pits forming in the metal surface.

The formation of colonies constitutes the creation of local cathodes or anodes (Little *et al.* 1990). Non uniform or mosaic colonisation of a metal surface by a biofilm results in the formation of differential aeration cells, where areas under respiring colonies are depleted of oxygen, relative to the non-colonised areas of naked metal. The movement of aggressive ions such as chloride to anodic sites would also be prevented as would outward diffusion of metabolites and corrosion products. The association of microcolonies with each other will set up initial sites of anodes and cathodes under, between and within each of the microcolonies. Such initiation will lead to numerous sites of corrosion being set up in a very small proximity (microns). This physical association may create the initiation of corrosion sites that represents the corrosion cell formation which leads to pepper pot pitting.

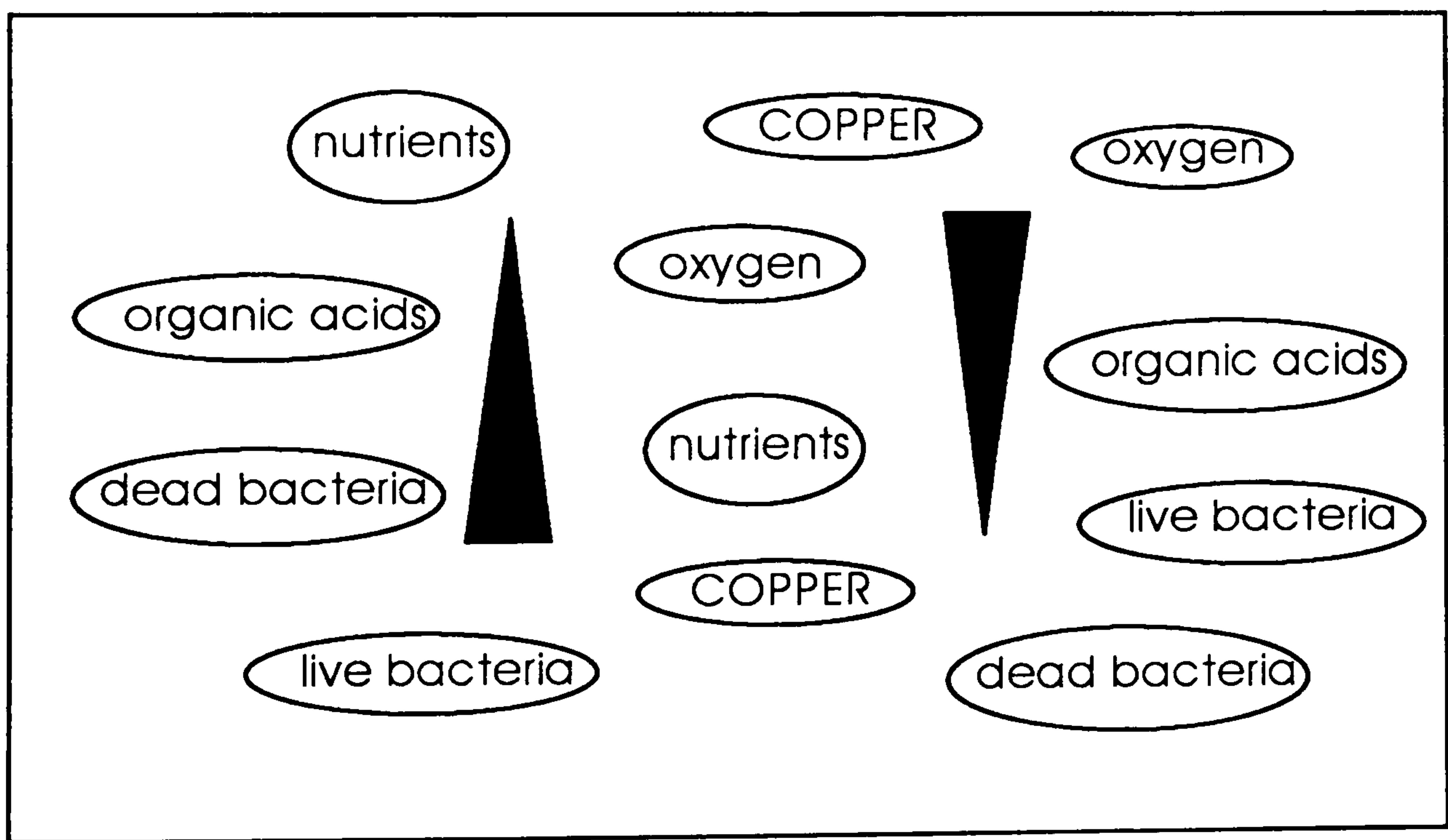


Figure 6.2 Schematic diagram of corrosion initiation and propagation on copper surfaces. Arrows indicate increasing and decreasing concentrations of components.

6.2.3 Role of polysaccharide in the corrosion process.

Copper ions will complex organic material such as humic acids and polysaccharide ligands in the presence of a biofilm. Exopolysaccharides were first demonstrated on copper surfaces by Chamberlain (1988). Biofilms have been demonstrated to support the dissolution of copper in the anodic reaction and so increase the rate of corrosion (Geesey *et al.* 1989). Furthermore the production of H^+ in neutral aqueous environments yields hydroperoxides (H_2O_2). Cu^+ reduces hydroperoxides, found extensively in corrosion products (Brown *et al.* 1992), which attack metabolic products (e.g. pyruvate) to form peracids which are known to be strong acids. It is these peracids which can oxidise Cu^+ , found mainly in the corrosion pits and at the interface between the copper surface and biofilm, to Cu^{2+} which has been detected mainly in the biofilm (Paradies, *et al.* 1992).

As other bacteria attach to the copper surface, grow and multiply the heterogeneous patchy layer of bacteria begins to form a mosaic of microcolonies on the surface. The mosaic microcolonies have a third lateral dimension to them as they grow out into the planktonic phase (one can presume this would enhance nutrient chelation). A fourth dimension is the physical growth of microcolonies resulting in death, recycling of nutrients and sloughing from the biofilm into the planktonic phase. It is the presence of the biofilm in water systems that are suspected for the reseedling of treated waters (pasteurisation and chlorination). Interaction between the biofilm and the water phase will occur as bacteria leave and enter each phase. Biofilm will also provide nutrients for amoeba and protozoa that will not only graze and to some extent control the biofilm but will increase the species diversity of the population.

In common with Cornwell *et al.* (1973) who demonstrated that removal of the carbon layer on the copper pipe reduced and prevented corrosion Fischer *et al.* (1992a) removed biofilm and reduced copper tube perforation after citric acid treatment but

only temporarily. Laboratory results indicated that the biofouling could be controlled by maintaining the hot water temperature greater than 55°C. From the results of the site surveys it was clear that not only was biofouling controlled but also that fewer copper tube failures occurred in hospitals operating their hot water systems at temperatures greater than 50°C. Therefore, even though the water contained a high concentration of AOC, and so had the ability to support microbial growth the bacteria were unable to form microcolonies and could not utilise this AOC or the dissolved oxygen concentration at the higher temperatures.

6.3 GROWTH OF LEGIONELLAE IN WATER AND IT'S IMPLICATIONS FOR THE SPREAD OF LEGIONELLOSIS

Legionellosis occurs through the dissemination of aerosols and immuno suppressed people are particularly at risk. In this investigation pathogenic bacteria such as *L. pneumophila* were of interest as this particular human pathogen has often been associated with water and surfaces of water circuits in hospitals. As the phenomena of pitting corrosion was occurring within the hot water circuit of a hospital the opportunity for this bacterium to be aerosolised was present although this particular route of infection had never been documented. The results demonstrate the ability of *L. pneumophila* to survive in environmental conditions similar to that in which corrosion was occurring (<50°), where increased temperatures (>55°C) not only control the presence of *L. pneumophila* but also control the presence of biofouling bacteria. Therefore the recommendations for the control of Legionella parallel those for the control of this particular type of corrosion. Where a protocol is instigated for the control of one the chances of the other occurring decrease.

In the hospital where corrosion was occurring, sections of the copper tube have been

replaced in alternative materials , for example plastic plumbing tubes. If the water system is operated at a temperature $> 55^{\circ}\text{C}$ then the total population including *L. pneumophila* will be controlled. However, if the temperature of the system cannot be controlled, then pronounced bacterial proliferation may occur where the temperature decreases to 50°C or less, as pasteurisation has no residual effect. The presence of plastic plumbing materials may exacerbate this growth. Results obtained in this study are therefore very important. Copper has been shown to possess bactericidal/static properties, while the plastic materials appears to result in an increased number of the water borne pathogen *L. pneumophila*. Although the results have demonstrated this phenomenon for only a short period of time the long term use of plastic material in systems will have to be monitored closely for biofouling.

6.4 FURTHER WORK

6.4.1 Influence of sulphate-reducing bacteria in the corrosion process

These bacteria were isolated from two of the sites where pepper pot pitting was occurring but their role in this process has been dismissed due to the low number that were detected and that other investigators did not detect sulphide. Previous investigations (Hamilton, 1985) have studied the growth of SRB in defined media or in open estuaries (Little, 1989). Extending the study to determine if they would grow in filter sterilised tap water as the sole media and carbon source may indicate if they have a role in pepper pot pitting.

6.4.2 Model plumbing system rigs

The use of rigs within the hospital where the corrosion was occurring would be of immense value. In some way this would take the role of the laboratory model closer

to a simulation of all the parameters involved in the biofouling process but this particular approach was out with the remit of this study. By operating an experimental rig apparatus the parameters of temperature, particulate matter, presence of bacteria could be separated to ascertain their individual emphasis on the phenomena of pepper pot pitting. The scaling up a laboratory model to an experimental rig has to take into consideration that problems of control, sterility and integrity will also be compounded. The rig would provide a truer representation of the scale of the water circuit.

6.4.3 Electrochemical testing

Although this particular study did not investigate corrosion mechanisms involved in this particular type of corrosion, the use of electrochemistry would be of immense value for the detection and quantification of microbial influenced corrosion of copper surfaces. Determination of the influence of bacterial microcolonies, particulate matter and temperature on the corrosion potential may have added strength to the argument of the holistic approach to the mechanism of pepper pot pitting. However, electrochemical techniques provide average readings for a surface area and therefore fail to provide information on localised corrosion that may be measured using recently developed techniques such as electrochemical impedance systems and electrochemical noise assays.

A number of other investigators have studied the involvement of polysaccharide pick up or chelation of copper. Recently Geesey and Bremner (1992) reported the production of polysaccharide by bacterium CC18 that actually protects copper surfaces by passivating the surface to prevent leaching of copper. Interacting this bacterium into a mixed population may provide useful information as to what happens when the polysaccharide of other bacteria are present i.e. would the passivation be stabilised or unstabilised? An ion chromatogram could be used to determine which

particular species of copper is taken up by which particular polysaccharides from which bacteria.

6.4.4 Atomic Force Microscopy

Having studied surface topography with a number of techniques there is another format of microscopy becoming available to the microbiologist. Conventionally used to study oxidation at the metal surface this particular microscope may present two areas where it could be interest to biofouling and corrosion. Firstly this microscope is now being used by microbiologists to visualise bacteria and providing very interesting initial results. With its ability to study oxidation at the surface, comparison of different bacterial growth conditions and their effect on corrosion rates may be able to studied using this instrument.

6.4.5 Legionella and amoebae

The number *Legionella pneumophila* recovered from the culture in the presence of copper and from the biofilms on the copper surface was, in general, less than what was recovered from the water phase in the presence of, and from the surface of plastic plumbing tube materials. Rowbotham (1980) has indicated that this water borne human pathogen is an intracellular organisms, and suggests that this may be an obligatory mechanism of survival. Although amoeba have not been studied in this present investigation, it would be interesting to determine whether the different material have an effect on the growth of the amoeba. For example, if copper was to have a negative effect on the growth of the amoeba then such results explain the decrease in recovery of *Legionella pneumophila* observed in this study and may support the work of Rowbotham (1980)

6.5 CONCLUDING REMARKS

From the site surveys and laboratory experiments that have been carried out there does appear to be a role for bacteria in pepper pot pitting. A whole range of environmental and biological factors determines whether or not this type of corrosion will occur or continue.

Each factor involved in corrosion and biofouling is a single entity that may or may not interact having different effects on either bacterial growth or the metallic surface.

Corrosion is composed of a series of anodic and cathodic reactions that will readily occur in the absence of bacteria and can be affected by the presence water, particulate matter on the pipe surface, other metals such as zinc, iron, welded joints and annealment.

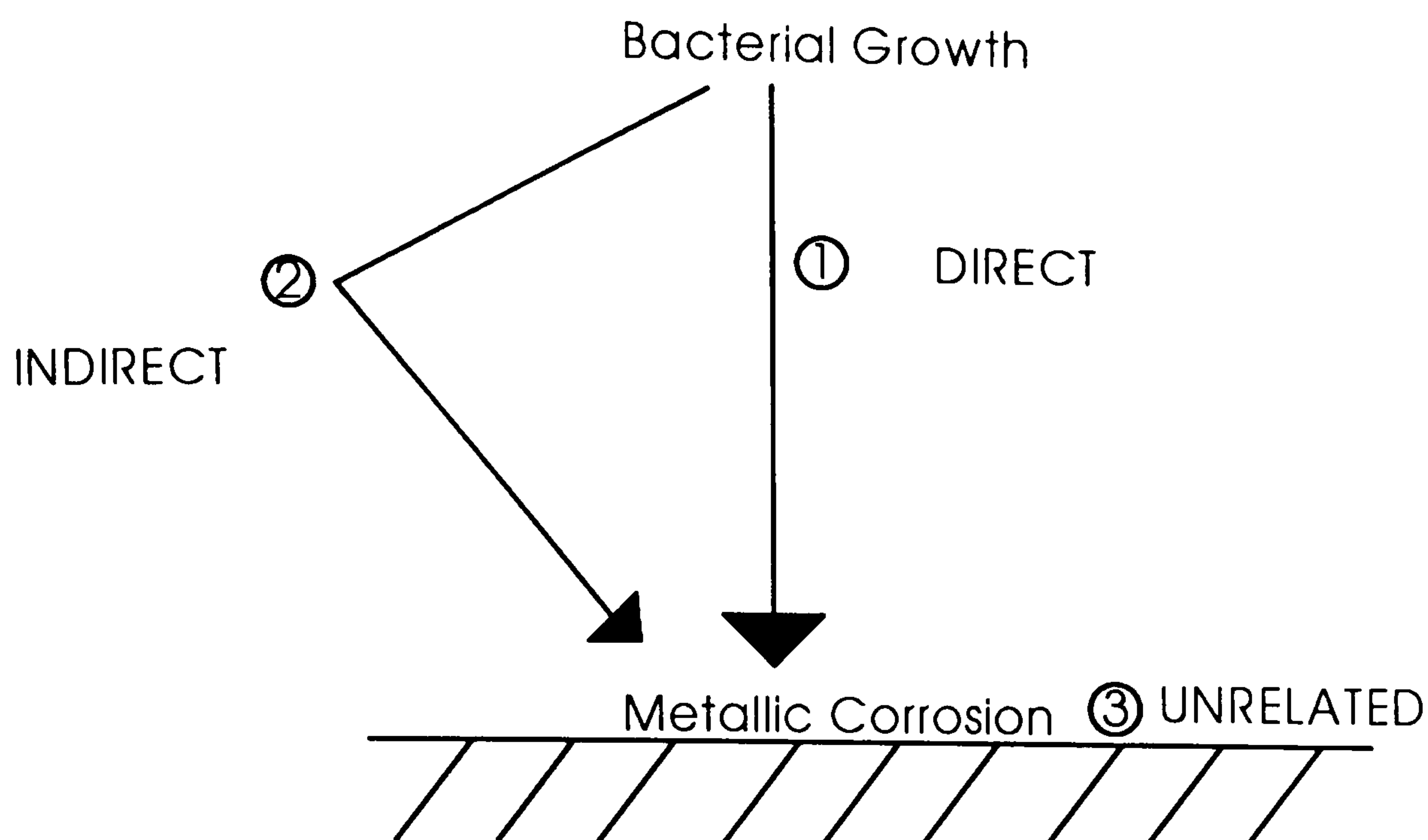
Biofilms represent the attachment of bacteria to a surface and growth into microcolonies with the resultant production of metabolic acids, exopolysaccharide and creation of concentration gradients when consumption is greater than transportation. In the water phase the physical flow or convective transport of solutes and nutrients are controlled in bulk phase by the flow rate. At the surface, in particular the viscous sublayer, movement of substrates and metabolic products are controlled by molecular diffusion. This in turn controls the growth of the bacteria directly in the 40 μm layer. As the biofilm matures and becomes established over a greater area of the pipe surface it will start to have an effect on fluid dynamics and heat dissipation.

Another entity that combines both the above parameters is heterogeneity. The metallic copper surface will be fabricated with minute inherent purities with grain

boundaries, milling lines presenting heterogeneous sites at which corrosion may manifest. It has been stressed that the biofilm itself is composed of discrete microcolonies forming a heterogeneous mosaic in the horizontal mode. Also discussed earlier is the lateral dimension of biofilms, providing sites of varying height and depth with the biofilm stack being thicker at their base providing strength and rigidity to stacks that have been found to protrude up to $100\mu\text{m}$ from the surface.

It has been suggested by some authors that heterogeneity may disappear as a biofilm matures, thickens and reach steady state (Videla, 1992) although there was no evidence of confluent biofilms in this present study even after developing biofilms for after 2 years. Some investigators of biofilms have modelled such systems using dead pan depth simulators. Wimpenny *et al* (1988) produced a model to simulate a $300\mu\text{m}$ deep biofilm, Gilbert *et al.* (1991) has modelled uniform biofilms impelled onto a filter, for growth rate studies and Angell and Chamberlain (1991) utilised a defined medium to provide uniform biofilm on copper surface. Importantly the investigator has to define what is one attempting to study.

In this particular study of copper tube corrosion the growth of mixed microbial communities on the copper surfaces was examined with water from a domestic water system as the sole carbon and energy source. In contrast to the above research groups (except Angell *et al.* (1991) who later switched to an artificial water with no carbon source) who developed homogenous biofilms from a monolayer up to $300\mu\text{m}$ all the biofilms formed using our laboratory rigs using either 1 cm^2 coupons or section of copper pipe exhibited this heterogeneous mosaic nature that continued for a period up to 2 years.



There are a number of affects that the presence of bacteria can have on corrosion of a metal surface. Firstly there may be no interaction or involvement of bacteria in the corrosion process. Alternatively the corrosion reaction can be influenced due to the production of metabolic acids, metal pickup by exopolysaccharides, creation of concentration cells, all of which will increase the corrosion potential. Bacteria however can become directly involved in the corrosion process through metabolic activity where autotrophic bacteria use anodic reaction products as an energy source. It is the latter two reactions where bacterial biofilms either influence and or induce corrosion of the copper surface that we have used to postulate how the corrosion has occurred. Primarily the bacteria will play a role in the initiation of corrosive sites within a very localised and confined environment due to the presence of numerous microcolonies on the surface. As the biofilm matures, enlarges and grows it no longer influences the corrosion process in the pits directly below the microcolony but bacterial will slough to resettle else where in the pipe line. Importantly the pit formation will then be driven as the corrosion potential increases with the likely event that perforation will eventually occur.

With the presence of particulate matter creating an increased surface area for bacterial growth and also creating concentration cells, the likely hood of pitting would be

increased. The number of initiated sites would therefore be increased in the presence of both bacterial microcolonies and particulate matter leading to the formation of multiloci, that would initiated corrosion sites to create pepper pot-pitting.

CHAPTER 7.0

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